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NOTE

Isolation of *Cryptococcus neoformans* var. *grubii* (serotype A) from Pigeon Droppings in Seoul, Korea

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Seventy-two pigeon dropping samples were collected from 26 different localities in Seoul and investigated for the occurrence of *Cryptococcus neoformans*. Seventeen samples from 8 different localities were found to be positive for *C. neoformans*. All isolates were obtained from withered pigeon droppings. Identification and serotyping of the isolates were determined by means of serological testing and DNA fingerprinting. All isolates belonged to *C. neoformans* var. *grubbi* (serotype A).

Key words: *Cryptococcus neoformans*, pigeon droppings, serotype A

Cryptococcus neoformans is an encapsulated, basidiomycetous yeast, which can cause life-threatening meningoencephalitis in immunocompromised individuals, especially those with AIDS (Mattsson *et al.*, 1999). Cryptococcosis is caused by inhalation of aerosols of soils and avian droppings containing fungal spores (Ellis and Pfeiffer, 1990).

Based on the antigenic composition of its polysaccharide capsule and biochemical characteristics, *C. neoformans* has been subdivided into three varieties, with 4 serotypes: *C. neoformans* var. *grubii* (serotype A), *C. neoformans* var. *neoformans* (serotype D) and *C. neoformans* var. *gatti* (serotypes B and C) (Wilson *et al*, 1968; Franzot *et. al.*, 1999). The former two varieties are distributed worldwide, whereas *C. neoformans* var. *gatti* is restricted to tropical and subtropical areas (Bennett *et al.*, 1977; Kohno *et al.*, 1994). In pathogenicity, *C. neoformans* serotypes A and D are responsible for most cases of cryptococcosis in immunocompromised and AIDS patients (Speed and Dunt, 1995). Serotype A is the major predominant clinical isolates of *C. neoformans* worldwide, whereas serotype D is most prevalent in some geographic areas (Dromer *et al.*, 1996; Criseo and Gallo, 1997; Tortorano *et al.*, 1997; Steenbergen and Casadevall, 2000). In terms of ecological distribution, *C. neoformans* var *gatti* has been associated with numerous eucalyptus species in tropical areas, whereas *C. neoformans* var. *grubii* and var. *neoformans* have been found in a variety of other environmental sources, such as avian droppings, soil and vegetables (Hsu *et al.*, 1994; Emmons, 1995; Lopez-Martinez and Castanon-Olivares, 1995). Avian droppings have been reported as a potential environmental sources of human pathogenic yeasts (Mattsson *et al.*, 1999). Pigeon droppings, which are abundant in public areas, could especially be a potential carrier in the spread of pathogenic yeasts into the environment and subsequently humans.

Pigeon droppings have been reported as the major environmental source of *C. neoformans* in several countries (Yehia, 1999; Kielstein *et al.*, 2000; Yimtubezenash *et al.*, 2001), but in Korea, there have been very limited studies on the occurrence and identification of the *C. neoformans* from pigeon droppings in public areas. Therefore, the isolation and identification of yeast from pigeon droppings in Korea might provide useful information for ecological and epidemiological studies of *C. neoformans*. In the present study, the potential health hazard posed by the occurrence and identification of *C. neoformans* in pigeon droppings were investigated in samples collected from public areas in Seoul.

Seventy-two pigeon droppings samples were collected from 26 different localities in the Seoul areas. The strain American Type Culture Collection (ATCC) 2344 was used as a reference strain of *C. neoformans*. Samples of pigeon droppings were harvested in sterilized tubes and transferred to the laboratory the same day. Approximately 5 g of pigeon dropping were suspended in 15 ml of sterilized distilled water by vortexing, and allowed to settle for 10 min. Aliquots (0.5 ml) of the supernatant from each

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sample were streaked onto Nigerseed (Guizotia abyssinica) agar plate, containing penicillin and streptomycin. The plates were incubated in the dark at 30°C for 8 days, but examined daily to observe for appearance of tiny dark brown pigmented colonies, suspected as being *C. neoformans*. All suspected colonies were segregated using sterilized tooth picks, and subcultured and maintained on Sabouraud dextrose agar (SDA) plates at 30°C. India ink preparations of the isolates were prepared to visualize the presence of capsules of cell. The isolates were identified by checking for growth on the SDA plates at 37°C and with the urease reaction on urea agar. The phenol oxidase activity was evaluated using DL-3, 4-dihydroxyphenylalanine (DL-DOPA) as substrate (Kabasawa *et al.*, 1991). The isolates were further identified by a carbohydrate assimilation and fermentation test using an API 20 C test kit (BioMerieux, USA). Growth of isolates on canavanine-glycine-bromothymol blue (CGB) agar was used to differentiate *C. neoformans* var. *neoformans* and var. *grubii* from *C. neoformans* var. *gatti*. The serotypes of isolates were determined using the factor sera slide agglutination test employing the Crypto Check test kit (Iatron Laboratories, Japan).

Molecular typing of isolates was carried out using PCR with $(GACA)_4$ and M-13 primer (5'-CGCCAGGGTTTT-CCCAGTCACGAC-3'). *C. neoformans* DNA was isolated using the phenol extraction method. Isolates were grown in malt extract broth at 26°C for 2 days, and harvested by centrifugation. Pellet was suspended in extraction buffer (50 mM Tris-HCl, 150 mM NaCl, 100 mM EDTA, 5% SDS, pH 8.0) and vortexed with iron beads (1.8 mm diam.) for 10 min. After treatment with proteinase K, the tube was incubated at 60°C for 30 min. After centrifugation, the supernatant was transferred to a new tube, treated with an equal volume of PCI (25 phenol: 24 chloroform: 1 isoamylalcohol) solution and mixed gently. After centrifugation, the upper phase was recovered and the DNA precipitated by the addition of 2 vol of ethanol. For PCR, 50 μl of reaction mixture, containing 100 pmole of $(GACA)_4$ and M-13 primer, 4 unit of *Taq* polymerase (Promega Inc, USA), 10 × buffer, 0.2 M dNTP mixture, and 50 ng of DNA template, was prepared. The amplification conditions were as follows; initial denaturation at 94°C for 5 min., followed by 30 cycles of denaturation at 94°C for 1 min., annealing at 42°C for 1 min. and extension at 72°C for 2 min. The PCR products were visualized using gel electrophoresis on a 1.5% agarose gel.

Of the 72 samples, 17 plates showed tiny dark-brown colonies on Nigerseed agar. All suspected isolates were grown on SDA at 37°C, and were shown to be positive with the urease test. India ink preparations of the yeast cells showed a polysaccharide capsule zone around the cells. Based on an assimilation profile of the API 20 kit test, the biochemical characteristics of all isolates corresponded to those of the *C. neoformans* reference strain. However, there was no color change on CGB agar plate, indicating the isolates were not *C. neoformans* var. *gatti*.

The prevalence of the serotypes of *C. neoformans* from pigeon droppings varies between countries. In Spain and Germany, serotype A is the most prevalent environmental isolate, such as in pigeon droppings, with the occurrence also of serotype D (Mitchell and Perfect, 1995; Kielstein and Bocklish, 2000). In southern Italy, only serotype A has been reported from pigeon dropping samples (Criseo and Gallo., 1997). In China, serotypes A and A/D have been reported from pigeon dropping samples (Li *et al.*, 1993). In this study, serotyping using the sera slide agglutination test demonstrated all isolates belonged to serotype A, indicating that this was the predominant serotype of *C. neoformans* from pigeon droppings in the Seoul area (Table 2).

Molecular markers based on PCR have been widely used to identify microorganism (Park *et al.*, 2003; Yoon *et al.*, 2003). DNA fingerprinting with minisatellite and a simple repeat sequence provided an especially suitable molecular marker for genetic identification and differentiation at various levels, ranging from species to individuals in human pathogenic fungi (Aufauvre-Brown *et al.*, 1992; Schonian *et al.*, 1993). PCR products with $(GACA)_4$ and

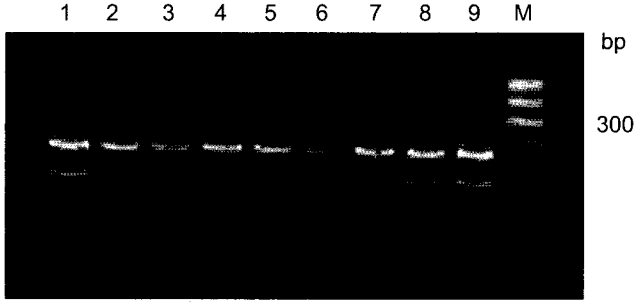

Fig. 1. PCR-based DNA fingerprints obtained by amplification of genomic DNA from strains of *Cryptococcus neoformans*, using the $(GACA)_4$ primer. M: Molecular markers. 1, KY101; 2, KY103; 3, KY105; 4, KY107; 5, KY108; 6, KY110; 7, KY112; 8, KY115; 9, ATCC2344.

Fig. 2. PCR-based DNA fingerprints obtained by amplification of genomic DNA from strains of *Cryptococcus neoformans*, using the (M13) primer. M: Molecular markers. 1, KY101; 2, KY103; 3, KY105; 4, KY107; 5, KY108; 6, KY110; 7, KY112; 8, KY115; 9, ATCC2344.

Table 1. Frequency of *Cryptococcus neoformans* isolated from pigeon droppings from different locations in Seoul, Korea

Location	District	No. of sample	Number of positive sample
Jongmyo Park	Jongno-gu	3	2
Seoul City Hall	Jongno-gu	3	2
Myeongnyun-dong	Jongno-gu	2	0
Marronnier Park	Jongno-gu	3	0
Gyeongbokgung (Palace)	Jongno-gu	2	0
Jangchung Park	Jung-gu	3	2
Namsan (Mt.)	Jung-gu	3	0
Hongeun-dong	Seodaemun-gu	2	1
Seoul Station	Seodaemun-gu	2	0
Hapjeong-dong	Mapo-gu	3	2
Sangam-dong	Mapo-gu	2	0
Hangang Park Office	Mapo-gu	4	0
Yeongdeungpo Station	Yeongdeungpo-gu	2	0
Yeongdeungpo Market	Yeongdeungpo-gu	4	0
Jinmyeong Girls' High School	Yangcheon-gu	3	0
Yangjae-dong	Seocho-gu	2	0
Seongnae-dong	Songpa-gu	4	2
Jamsil-dong	Songpa-gu	2	0
Olympic Park	Songpa-gu	4	0
Boramae Park	Dongjak-gu	4	3
Chungang University	Dongjak-gu	2	0
Jeongneung-dong	Seongbuk-gu	2	0
Children's Grand Park	Gwangjin-gu	3	3
Taereung	Jungnang-gu	3	0
Hongneung	Dongdaemun-gu	2	0
Yeokchon-dong	Eunpyeong-gu	3	0

M-13 primer have demonstrated different band patterns between *Cryptococcus* species (Meyer *et al.*, 1993). Therefore, these primers are considered useful molecular markers for the identification of *Cryptococcus* at the species level. In this study, in the analysis of the PCR products amplified with $(GACA)_4$ and M-13 primer, all isolates showed identical band patterns, indicating all isolates belonged to the same species (Figs. 1 and 2).

All the isolates of *C. neoformans* used in this study were obtained from old withered pigeon droppings, not fresh droppings, which was in accordance with the previous reports that *C. neoformans* was not found from fresh pigeon droppings or pigeon cloaca samples (Mishra *et al.*, 1981). These reports suggest that *C. neoformans* may not be a natural inhabitant of fresh pigeon dropping. Rather, pigeon droppings might be inoculated by cells of *C. neoformans* from environmental sources, such as contaminated soil or air, as they provide a good nutrient source for the growth of yeast (Casadevall and Perfect, 1998).

In our previous report, the occurrence of *C. neoformans* was investigated in pigeon droppings collected from only

Table 2. Locations and serotypes of *Cryptococcus neoformans* isolated in Seoul, Korea

Strain Number	Location	Serotype
KY101	Jongmyo Park	A
KY102	Jongmyo Park	A
KY103	Seoul City Hall	A
KY104	Seoul City Hall	A
KY105	Jangchung Park	A
KY106	Jangchung Park	A
KY107	Hongeun-dong	A
KY108	Hapjeong-dong	A
KY109	Hapjeong-dong	A
KY110	Seongnae-dong	A
KY111	Seongnae-dong	A
KY112	Boramae Park	A
KY113	Boramae Park	A
KY114	Boramae Park	A
KY115	Children's Grand Park	A
KY116	Children's Grand Park	A
KY117	Children's Grand Park	A

three localities in Seoul (Chee and Kim, 2003). In this study, pigeon dropping samples from 8 out of 26 localities (30.4%) were shown to be positive for *C. neoformans*, with the 8 localities in question covering wide areas, including 6 districts of Seoul (Table 1). These results demonstrate that pigeon droppings are an important and environmentally widespread source for *C. neoformans* in areas of Seoul. All our isolates were found in public areas, such as city parks and streets, suggesting a potential public health hazard due to human exposure to airborne cells of *C. neoformans* in public areas. Further studies, covering wider areas of the Korean peninsula, with high numbers of samples, will be needed to investigate the geographic distribution and population structure of *C neoformans* from pigeon droppings, as well as the possible associations of disease with exposure to environmental sources of *C. neoformans*, such as pigeon droppings.

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