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

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**Three hundred and sixty five samples of avian droppings, collected from parks and zoo, were investigated for the occurrence of *Cryptococcus neoformans* in Korea. Thirteen samples were positive for *C. neoformans*. All isolates were obtained from withered pigeon droppings. Identification and serotyping of isolates were determined by means of serological test and polymerase chain reaction (PCR) fingerprinting. All isolates belonged to *C. neoformans* var. *grubbi* (serotype A).**

**KEYWORDS:** ***Cryptococcus neoformans*, Pigeon droppings, Serotype A**

*Cryptococcus neoformans* is an encapsulated, basidiomycetous yeast-like fungus that can cause meningoencephalitis in immunocompromised individuals, particularly AIDS patients (Mattsson *et al.*, 1999). Individual is thought to be infected by inhalation of airborne fungal cell from environmental sources (Ellis and Pfeiffer, 1990).

On the basis of the antigenic composition of its polysaccharide capsule and biochemical differences, *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* (serotype B and C) (Franzot *et al.*, 1999; Wilson *et al.*, 1968). Former two varieties have a worldwide distribution, whereas *C. neoformans* var. *gatti* is restricted to tropical and subtropical areas (Bennett *et al.*, 1977; Kohno *et al.*, 1994). In pathogenicity, it has been reported that *C. neoformans* serotype A and D are the causative agents of cryptococcosis in immunocompromised or AIDS patients while *C. neoformans* var. *gatti* is associated with the infection of individual with normal immune status (Speed and Dunt, 1995). Serotype A is the predominant majority of clinical isolates of *C. neoformans* throughout the world, whereas serotype D is prevalent in some geographic areas (Criseo and Gallo, 1997; Dromer *et al.*, 1996; Steenbergen and Casadevall, 2000; Tortorano *et al.*, 1997). In terms of ecological distribution of *C. neoformans*, *C. neoforamns* var *gatti* has been isolated from *Eucalyptus* in tropical areas whereas *C. neoformans* var. *grubii* and var. *neoformans* have been found in a variety of environmental sources such as avian droppings, soil, fruits, and vegetables (Emmons, 1995; Hsu *et al.*, 1994; Lopez-Martinez and Castanon-Olivares, 1995). In several countries, avian droppings, especially pigeon droppings, have been considered to be the major environmental source of *C. neoformans* serotype A and D (Kielstein *et al.*, 2000; Mahmoud, 1999; Yimtubezenash *et al.*, 2001). The reason for the high occurrence of *C. neoformans* in avian excreta is believed to be due to a utilization of nitrogenous compounds such as uric acid and creatinine in excreta (Lattman and Walter, 1968). In Korea, the distribution of serotype of the *C. neoformans* has not been previously reported. In this study we examined the occurrence and serotypes of *C. neoformans* in avian droppings.

## Materials and Methods

A total of 365 samples including 275 pigeon droppings and 90 zoo bird droppings were collected from parks and zoo in Korea (Table 1). A strain ATCC (American Type Culture Collection) 2344 was used as a reference strain. Samples of droppings were harvested in sterilized tube and transfered to the laboratory on the same day. Samples were suspended in sterilized distilled water at a ratio of 1 : 5 by vortexing and allowed to settle for 20 min. From the supernatant, 2 m*l* of aliquot from each tube were inoculated onto Nigerseed (*Guizotia abyssinica*) agar plate containing penicillium and streptomycin. The plates were incubated in the dark at 26°C for 8 days. The plates were examined daily to observe for appearance of the brown colored yeast form colonies, suspected *C. neoformans*. All suspected colonies were picked out using sterilized toothpicks and subcultured on SDA (Sabouraud dextrose agar) plate at 26°C for maintenance. India ink preparation of isolates was made to visualize the presence of capsule. The isolates were identified by checking for its growth on SDA at 37°C and urease reaction on urea agar. The isolates were further identified by carbohydrate assimilation and fermentation test by API 20 C test kit (bioMerieux, Hazelwood). Growth of isolates on CGB (canavanine-gly-

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**Table 1.** Frequency of *Cryptococcus neoformans* var. *grubii* isolated from avain droppings from different localities

| Locality | Avian | No. of samples | No. of positive sample | Isolate No. |
|---|---|---|---|---|
| Borame park, Seoul | Pigeon | 25 | 1 | K006 |
| Han-gang city park, Seoul | Pigeon | 65 | 3 | K112, K132, K138 |
| Seoul children park, Seoul | Pigeon | 35 | 2 | K152, K160 |
| Dalsung park, Tae-Gu | Pigeon | 20 | 1 | K041 |
| Bo-Mun Mountain park, Taejeon | Pigeon | 50 | 3 | K079, K081, K093 |
| Seo Taejeon bird park. Taejeon | | | | |
| Park area | Pigeon | 80 | 3 | K190, K202, K208 |
| Bird cage | Turkey | 15 | 0 | |
| | Parrot | 15 | 0 | |
| | Peacock | 15 | 0 | |
| | Silver pheasant | 15 | 0 | |
| | Pheasant | 15 | 0 | |
| | Gold pheasant | 15 | 0 | |

cine-bromothymol blue) agar was used to differentiate *C. neoformans* var. *neoformans* and var. *grubii* from *C. neoformans* var. *gatti*. The serotypes of isolates were determined by factor sera slide agglutination test using the Crypto Check test kit (Iatron labs Inc., Tokyo, Japan). The serotypings were carried out twice.

Molecular typing of isolates was carried out using PCR (polymerase chain reaction) with $(GACA)_4$ primer. *C. neoformans* DNA was isolated by using phenol extraction method. Isolates grown in malt extract broth at 26°C for 5 days were 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 2 min. After treatment of proteinase K, the tube was incubated at 60°C for 30 min. After centrifugation, supernatant was transfered to new tube and treated with the equal volume of PCI (25 phenol: 24 chloroform: 1 isoamylalcohol) solution and then mixed gently. After centrifugation the upper phase was recovered and the DNA was precipitated by adding 2 volume of ethanol. For PCR, a total of 50 $\mu l$ of reaction mixture contained 50 pmole of $(GACA)_4$ primer, 4 unit of *Taq* polymerase (Promega Inc. Madison, WI), 10× buffer, 0.2 M dNTP mixture, and 50 ng of DNA template. Amplification condition was an 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 1 min. PCR products were visualized using gel electrophoresis on a 1.5% agarose gel.

## Results and Discussion

Of the total 365 samples, dark-brown yeast form colonies were observed on Nigerseed agar on thirteen cultures. All suspected isolates were grown on SDA at 37°C and showed positive urease test. India ink preparation of yeast cell showed polysaccharide capsule zone around cell. On the basis of assimilation profile on API 20 kit test, biochemical characteristics of our isolates corresponded to those of *C. neoformans* reference strain. The growth of isolates with media color change was not observed on CGB agar plate, indicating that isolates are not *C. neoformans* var. *gatti*.

Avian droppings and soils contaminted with these have been known to be a good nutrient source for growth of *C. neoformans* (Casadevall and Perfect, 1998). There have been many reports on the varying of occurrence of *C. neoformans* in avian droppings (Hsu *et al.*, 1994; Khorsavi *et al.*, 1997; Kielstein *et al.*, 2000; Yimtubezenash *et al.*, 2001). In this study, all isolates were obtained from pigeon droppings, demonstrating that pigeon dropping is the prinicipal environmental sources for *C. neoformans* in Korea. Littman and Brook (1968) claimed that pigeon was natural carrier of *C. neoformans*. In present study, all the colonies were obtained from withered old pigeon droppings, not from fresh droppings. This is in accordance with the reports that old accumulated pigeon excretas are appropriate source for the isolation of *C. neoformans* (Emmons, 1995; Khosravi, 1997). It has been reported that *C. neoformans* was not found from fresh pigeon droppings and pigeon cloaca samples (Mishra *et al.*, 1981). These findings may suggest that *C. neoformans* may not be common natural inhabitant in pigeon dropping. Rather, pigeon dropping might be inoculated by propagule of *C. neoformans* from other environmental sources such as contaminated soil or air, and then provide good nutrient sources for the growth of yeast.

It has been reported that *C. neoformans* was isolated from zoo bird excreta other than pigeon in zoos of Mexico and Germany (Bauwen *et al.*, 1986; Lopez-Martinez and Castanon-Olivares, 1995). Our excreta samples of zoo bird in the City Zoo of Taejeon, however, did not give the positive result of *C. neoformans*. In the City Zoo of Taejeon, *C. neoformans* was found in pigeon droppings at park area whereas no *C. neoformans* was isolated from zoo bird droppngs in bird cage. This fact showed that the

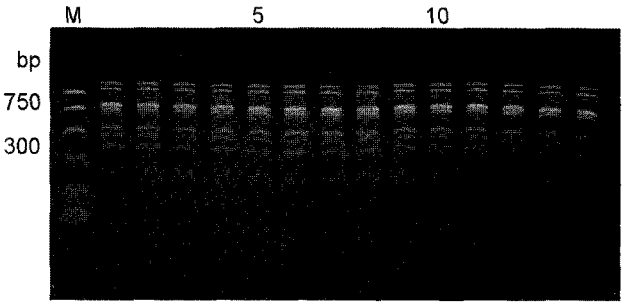


**Fig. 1.** Agarose gel electrophoresis of PCR product with $(GACA_4)$ primer. M : Molecular marker, 1 K006, 2. K041, 3. K079, 4. K081, 5. K093, 6. K112, 7. K132, 8. K138, 9. K152, 10. K160, 11. K190, 12. K202, 13. K208, 14. ATCC 2344.

occurrence of *C. neoformans* between pigeon droppings and other avian droppings was different in same area. This might be due to little chance of accumulation of withered faeces in bird cage since bird cage in zoo is cleaned everyday.

Serotyping by sera slide agglutination test demonstrated that isolates agglutinated with factor serum 1 and 7, indicating that all isolates belonged to serotype A. In the analysis of PCR products amplified with $(GACA)_4$ primer, all isolates showed a uniform banding patterns, representing that isolates belonged to the same species and serotype (Fig. 1). Prevalence of serotype of *C. neoformans* varied among countries. In Spain and Germany, serotype A is prevalent in the environmental isolates with the occurrence of serotype D (Kielstein and Bocklish, 2000; Mitchell and Perfect, 1995). In clinical samples, serotype A is predominant in most countries except some areas of Europe (Mitchell and Perfect, 1995; Steenbergen and Casadevall, 2000). In this study, only serotype A was found in Korea. This result may suggest that serotype A is predominant environmental serotype in Korea. Considering limited number of samples and localities, however, the fact that other serotypes was not observed does not prove an absence of other serotypes from pigeon droppings in Korea. Further studies covering more wide areas with high number of samples will be needed to examine the population structure of *C. neoformans* in Korea. In other east Asian countries such as Japan and Taiwan, only serotype A was reported from clinical samples (Hsu *et al.*, 1994; Kohno *et al.*, 1994). Serotyping of Korean clinical samples was not reported. Pigeon excreta have long been known to be associated with a possible source for human infection of *C. neoformans* (Garcia-Hermos *et al.*, 1997). Thus, serotyping of *C. neoformans* isolates is critical to elucidate the route of human infection and to reveal possible infection source. Future studies are needed to investigate the serotype of Korean clinical samples and possible association of disease with environmental source of *C. neoformans*.

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