Isolation of Cryptococcus neoformans var. grubii (serotype A) from Pigeon Drop-
pings in Korea

Hee Youn Chee* and Yoon Kyoung Kim
Division of Biological Sciences, Medical School, Konyang University, Non-San, Chung-Nam, South Korea
(Received February 7, 2003)

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. grubii (serotype A).

KEYWORDS: Cryptococcus neoformans, Pigeon droppings, Serotype A

Cryptococcus neoformans is an encapsulated, basidiomy-
cetous yeast-like fungus that can cause meningoencephali-
tis 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. neofor-
mans var. gatti (serotype B and C) (Franzot et al., 1999;
Wilson et al., 1968). Former two varieties have a world-
wide 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 immunocomprom-
ised 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. neoform-
ans throughout the world, whereas serotype D is preva-
ient 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. neoformans var. gatti has been iso-
lated from Eucalyptus in tropical areas whereas C. neofor-
mans var. grubii and var. neoformans have been found in
a variety of environmental sources such as avian drop-
pings, soil, fruits, and vegetables (Emmons, 1995; Hsu et
In several countries, avian droppings, especially pigeon
droppings, have been considered to be the major environ-

---

*Corresponding author <E-mail: hychee@kytis.konyang.ac.kr>

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. Sam-
ple 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 ml 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 tooth-
picks 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 iso-
lates were further identified by carbohydrate assimilation
and fermentation test by API 20 C test kit (bioMerieux,
Hazelwood). Growth of isolates on CGB (canavanine-gly-
cine-bromothymol blue) agar was used to differentiate \textit{C. neoformans} var. \textit{neoformans} and var. \textit{grubii} from \textit{C. neoformans} var. \textit{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), primer. \textit{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 then mixed gently. After centrifugation the upper phase was recovered and the DNA was precipitated by adding 2 phenol: 24 chloroform: 1 isoamylalcohol) solution and new tube and treated with the equal volume of PCI (25 µl of reaction mixture contained 50 pmole of (GACA), primer, 4 unit of \textit{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 \textit{C. neoformans} reference strain. The growth of isolates with media color change was not observed on CGB agar plate, indicating that isolates are not \textit{C. neoformans} var. \textit{gatti}.

Avian droppings and soils contaminated with these have been known to be a good nutrient source for growth of \textit{C. neoformans} (Casadevall and Perfect, 1998). There have been many reports on the varying of occurrence of \textit{C. neoformans} in avian droppings (Hsu et al., 1994; Khorsavi et al., 1997; Kielstein et al., 2000; Yimtubezash et al., 2001). In this study, all isolates were obtained from pigeon droppings, demonstrating that pigeon dropping is the principal environmental sources for \textit{C. neoformans} in Korea. Littman and Brook (1968) claimed that pigeon was natural carrier of \textit{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 excreta are appropriate source for the isolation of \textit{C. neoformans} (Emmons, 1995; Khosravi, 1997). It has been reported that \textit{C. neoformans} was not found from fresh pigeon droppings and pigeon cloaca samples (Mishra et al., 1987). These findings may suggest that \textit{C. neoformans} may not be common natural inhabitant in pigeon dropping. Rather, pigeon dropping might be inoculated by propagule of \textit{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 \textit{C. neoformans} was isolated from zoo bird excreta other than pigeon in zoos of Mexico and Germany (Bauwen et al., 1985; 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 \textit{C. neoformans}. In the City Zoo of Taejeon, \textit{C. neoformans} was found in pigeon droppings at park area whereas no \textit{C. neoformans} was isolated from zoo bird droppings in bird cage. This fact showed that the

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

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

References


