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Isolation and Characterization of *Cryptococcus neoformans* from Environmental Sources in Busan

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Twenty nine samples of pigeon droppings (n = 12) and soil contaminated with avian excreta (n = 19), collected from different sites in Busan, were examined for isolation and characterization of *Cryptococcus neoformans*. Of these samples, 5 strains of *C. neoformans* were recovered from pigeon droppings (5/12 : 41.7%). All isolates were belonged to *C. neoformans* var. *grubii* (serotype A). The extracellular enzyme activities of the strains by using the API-ZYM system showed two different enzymatic patterns. The genetic variability among *C. neoformans* isolates was analyzed by random amplified polymorphic DNA (RAPD) using three 10-mer primers. Two different RAPD patterns, which clearly distinguished the isolates, were identified. Analysis of RAPD patterns provided a good characterization of environmental strains of *C. neoformans* serotype A as a heterogeneous group and were in good agreement with enzymatic profiles.

KEYWORDS: API-ZYM, *Cryptococcus neoformans*, Pigeon droppings, RAPD

Cryptococcus neoformans is a basidiomycetous yeast-like fungus which causes respiratory and neurological disease in humans and animals. (Lewis *et al.*, 1972; Casadevall *et al.*, 1992). Recently *C. neoformans* was divided into three varieties; *C. neoformans* var. *grubii* (serotype A), *C. neoformans* var. *neoformans* (serotype D) and *C. neoformans* var.*gattii* (serotypes B and C) (Franzot *et al.*, 1999). These varieties of *C. neoformans* are easily differentiated by their biochemical properties and antigenic composition of capsular polysaccharides.

C. neoformans has a worldwide distribution and has been associated with a variety of environmental sources in particular, bird excreta and decaying wood (Walter *et al.*, 1968; Ruiz *et al.*, 1981). The association between *C. neoformans* and bird excreta, especially weathered pigeon dropping, has been confirmed in several studies but many questions related to the ecology and epidemiology of *C. neoformans* are still unanswered.

The most common isolate responsible for human infection is *C. neoformans* var. *grubii* serotype A (Ellis, 1987). *C. neofomrans* var. *gattii* is restricted predominantly to tropical and subtropical climates (Kwon-Chung *et al.*, 1984) and has been proposed to have a specific ecological association with a number of *Eucalyptus* species (Pfeiffer *et al.*, 1992). Attempts to correlate specific exposures to cryptococcal strains as defined the molecular backgrounds may help to address unanswered epidemiological questions.

Several typing approaches have been used in epidemiological studies, including serotyping, biotyping and polymerase chain reaction (PCR) fingerprinting. Among them, the serotyping schemes have been found to be important since the association of infection with serotype A strains in AIDS patients was confirmed by recent epidemiological and ecological studies (Varma *et al.*, 1995). Biotyping, particularly extracellular enzyme activity, has been recently proposed as a virulence determinant and pathogenicity of this fungus. Studies on this activity, together with genetic, serological and biochemical investigations, would be very useful to characterize different *C. neoformans* strains and to elucidate the epidemiology of cryptococcosis (Chen *et al.*, 1996; Vidotto *et al.*, 2005). PCR fingerprinting has been used as the major typing technique in the molecular epidemiologic survey of *C. neoformans* (Meyer *et al.*, 1993). Especially, random amplified polymorphic DNA (RAPD) fingerprinting has been demonstrated to have the ability to discriminate between closely related isolates within given population (Brandt *et al*, 1995).

The incidence of cryptococcal infection has increased in recent years as a result of a large increase in AIDS cases and the expanded use of immunosuppressive drugs (Currie *et al.*, 1994; Sorvillo *et al.*, 1997). Despite the increasing number of published studies on the ecology and epidemiology of *C. neoformans* isolates in the worldwide, there is not a noticeable information about the molecular epidemiology of cryptococcal infection in Korea.

In the present study, we investigated the environmental occurrence of *C. neoformans* from natural habitats, pigeon

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droppings and avian guano in Busan, and analyzed the biotypic and genetic characteristics of the isolates of *C. neoformans*.

Materials and Methods

Fungal isolation and identification. A total of 29 samples of pigeon droppings (n = 12) and soil contaminated with avian excreta (n = 17) were collected from different locations in the city of Busan over a 3-month period, from June to August 2002 (Table 1). Each sample was processed as described previously (Hwang, 2002). Esculin based medium (EBM) and bird seed agar (BSA) were used as a primary plating medium for detection of the typical brown pigmentation due to the activity of phenoloxidase of *C. neoformans*. All plates were incubated at 30°C for 2 to 10 days. All suspected brown colonies were picked and subcultured on Sabouroud Dextrose Agar (SDA) plates for identification. All isolates were first tested on the basis of their biochemical characteristics, such as the assimilation of sugars using API 20C AUX system (BioMerieux, Marcy-l'Etoile, France), positive urease activity on Christensen's urea broth, inability to reduce nitrate, and the ability to grow at 37°C. The biovariety study was performed by culturing the isolates on canavanine-glycine-bromthymol blue (CGB) medium to determine the use of glycine as a carbon source (Kwon-Chung *et al.*, 1982). A color change from light yellow green to covalt blue was considered a positive result for the CGB test, indicating *C. neoformans* var. *gattii* (serotype B/C), otherwise no color change was considered a negative result, indicating *C. neoformans* (serotype A/D). Serotyping was performed by a slide agglutination procedure using monoclonal antibodies specific for capsular polysaccharide (Crypto Check; Iatron Laboratories, Tokyo, Japan) according to the manufacturer's instructions.

Enzymatic activities. Extracellular enzyme activities of environmental *C. neoformans* isolates were tested by using API-ZYM system. The API-ZYM system (BioMerieux, Inc., France) is a micromethod that allows rapid determination of 19 enzymatic reactions. The API-ZYM strips were inoculated, incubated and interpreted according to the manufacturer's instructions. After inoculation each cupule of strips with 65 μl of a dense suspension (McFarland No. 5 or 6 standards) in water, the panel was incubated in air at 37°C for 4 h. The presence of enzyme activity was determined by the addition of color reagents and compared to a chart for the quantity of hydrolyzed substrate. Extracellular proteolytic activity was measured as described by Aoki *et al.* (1994), using Yeast Carbon Base (YCB) agar plates supplemented with 0.1% (w/v) bovine serum albumin (BSA) plus 0.01% (w/v) polypeptone (Pp). Extrcellular phospholipase activity was measured according to Polak (1992) using heart infusion agar plates supplemented with egg-yolk.

RAPD analysis. For the DNA extraction, chromosomal DNAs of all isolates were extracted using a method modified from those of Zhu *et al.* (1993) and Yamamoto *et al.* (1995). The strains were cultured in brain heart infusion broth for 48 h at 30°C and washed the cell twice with TE buffer (100 mM Tris-HCl pH 8.0, 1 mM EDTA). 100 μl of the sample pellet was mixed with 250 μl of extraction buffer (100 mM Tris-HCl pH 9.0, 40 mM EDTA), 50 μl of 10% sodium dodecyl sulfate, and 200 μl of benzyl chloride. After mixing with a vortex mixer and incubated at 50°C for 30 min with gentle shaking. After centrifugation at 6000 ×g at 4°C for 10 min, the supernatant was transferred to another eppendorf tube, 3 M sodium acetate was added at 1/10 volume of the aqueous supernatant, and the mixture was cooled at 0°C for 10 min. The DNA was precipitated with 250 μl of isopropanol (−70°C,

Table 1. Frequency of *Cryptococcus neoformans* isolated from different localities in Busan

Locality	Source	No. of samples	No. of isolated sample	Isolates No.
Geum-gang park	Pigeon droppings	2	1	CNS23
	Turkey cage	1	–	–
Geum-gang botanical garden	Pigeon droppings	2	–	–
Busan children park	Pigeon droppings	2	1	CNS24
	Parrot cage	2	–	–
Busan station	Pigeon droppings	2	1	CNS25
Gwang-an dong residential area	Avian excreta	2	–	–
Jang-jeon subway station	Pigeon droppings	2	1	CNS26
Soo-jeong dong residential area	Pigeon droppings	2	1	CNS27
Boo-gok dong residential area	Contaminated soil	3	–	–
	Decaying wood	2	–	
Dae-cheung dong residential area	Avian excreta	1	–	–
	Decaying wood	3	–	
Dang-gam dong pet shop	Avian excreta	1	–	–
Yong-doo mountain park	Contaminated soil	2	–	–

2 h) and then the precipitate was washed with 70% ethanol, dried and resuspended in 40 μl of TE buffer. The DNA concentration was determined with a UV spectrophotometer.

PCR was performed using an *AccuPower*©PCR Premix (Bioneer, Korea), containing 250 μM of each deoxyribonucleoside triphosphate, 40 mM of KCl, 10 mM of Tris-HCl (pH 9.0), 1.5 mM of $MgCl_2$, and 1 Unit of *Taq* DNA polymerase. 75 ng of genomic DNA and 1.0 μM of RAPD primer were then add to a PCR Premix tube. PCR was carried out in a final volume of 20 μl. The following three primers were used for the RAPD PCR analysis : OPH-20 (5'-GGGAGACATC-3'), OPH-02 (5'-TCGGACGTGA-3') and R-28 (5'-ATGGATCCGC-3'). These were prepared on the basis of the reports of Yamamoto *et al.* (1995). Amplification was performed for 30 cycles in a Perkin-Elmer 2400 thermal cycler as follows : 4 min of initial heat at 94°C, 45 s of denaturation at 92°C, 60 s of annealing at 34°C, and 90 s of primer extension at 72°C, followed by a final extension cycle for 10 min at 72°C. The amplification products were analyzed by electrophoresis on 2.0% agarose gels in 1× TAE (Tris-acetate-EDTA) buffer at 70 V for 75 min and then stained with ethidium bromide.

Results

Fungal isolation and identification. Out of the 29 samples, five *C. neoformans* were recovered from pigeon droppings. Dark-brown pigmentation in both EBM and BSA media, followed by microscopic examination of India ink preparations facilitated the identification of *C. neoformans* colonies. All of the suspected isolates were identified as *C. neoformans* based on the results of analysis using the API 20C kit system, their ability to grow at 37°C, and a positive urease test. All strains of *C. neoformans* were isolated from pigeon droppings, especially on their shelters and the prevalence was 41.7% (5/12). There was significant difference in isolation frequency between pigeon droppings and other samples (0/17). All five isolates of *C. neoformans* presented agglutination patterns consistent with their assignment to erotype A via Cryptochek system. All isolates were confirmed to be *C. neoformans* var. *grubii* serotype A on the basis of no color change on CGB medium and serotyping (Table 2).

Table 2. Results obtained with the testing media and serotyping of *C. neoformans*

Isolates No.	Testing media				Serotype
	BSA	Urea agar	37°C growth	CGB	
CNS23	+	+	+	–	A
CNS24	+	+	+	–	A
CNS25	+	+	+	–	A
CNS26	+	+	+	–	A
CNS27	+	+	+	–	A

*BSA, bird seed agar; CGB, canavanine-glycine-bromthymol blue.

Enzymatic activities. The extracellular enzyme activities of the environmental isolates of *C. neoformans*, determined by the API-ZYM system, protease and phospholi-

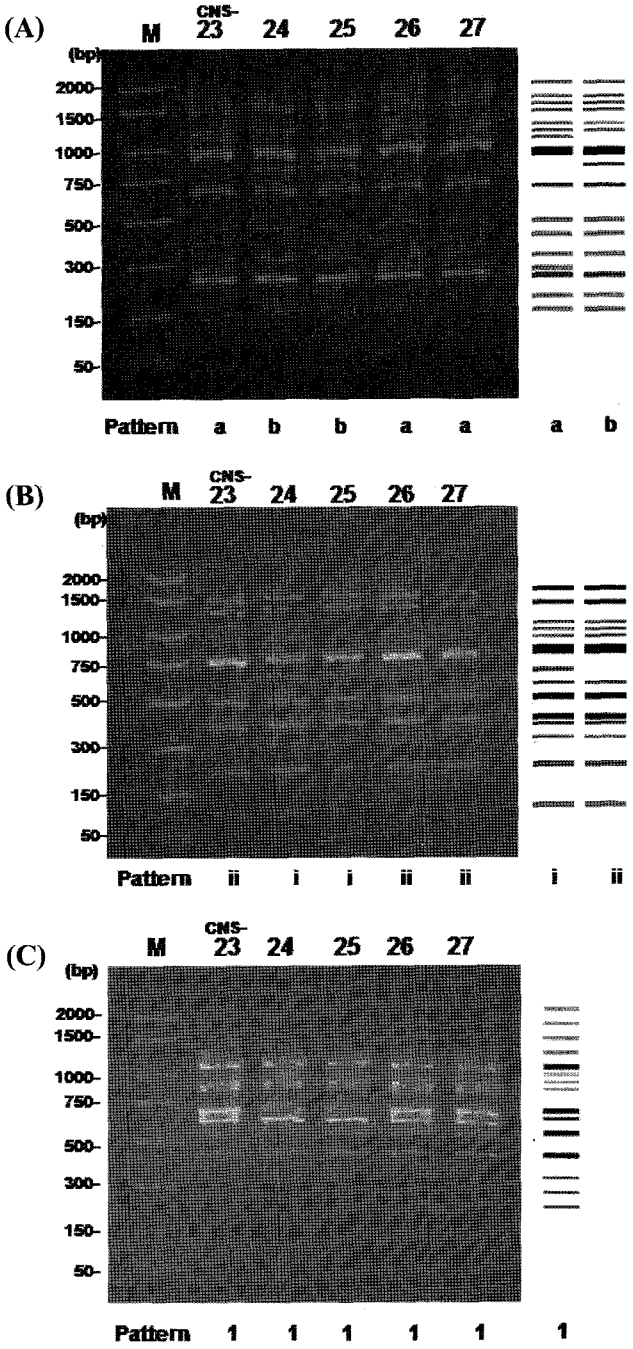

Fig. 1. RAPD profiles of *C. neoformans* serotype A amplified with the primers. (A) Primer OPH-20 : (B) Primer OPH-02 : (C) Primer R-28. Numbers at the top are strain numbers. Molecular size markers in base pairs are indicated on the left.

pase activities are summarized in Table 3. The enzymes produced by all isolates were alkaline phosphatase (No. 2), esterase C4 (No. 3), esterase lipase C8 (No. 4), leucine arylamidase (No. 6), acid phosphatase (No. 11), naphthol-AS-BI-phosphohydrolase (No. 12), α-glucosidase (No. 16), and β-glucosidase (No. 17). Only two isolates (CNS 24, 25) of *C. neoformans* produced N-acetyl-β-glucosaminidase (No. 18). The environmental isolates showed two distinguishable enzymatic profiles in the API-ZYM test. In addition, the *C. neoformans* isolates examined in duplicate showed no variation in their enzyme producing ability. All of the five isolates of *C. neoformans* were positive for the production of extracellular protease and phospholipase.

RAPD analysis. The RAPD fingerprint profiles of the 5 strains of *C. neoformans* serotype A obtained with the three different 10-mer primers are shown in Figs. 1 and 2. The RAPD profiles produced the identical RAPD patterns in all instances. Primer OPH-20 revealed major differences in banding patterns about 900 bp and 300bp, respectively, which distinguished the profiles a and b (Fig. 1A). Primer OPH-02 revealed two different profiles in banding pattern about 650 bp, which distinguished the profiles i and ii (Fig. 1B). Primer R28 showed no differences in banding patterns, deginated 1 (Fig. 1C). Table 2 summerized the RAPD profiles for all environmental strains of *C. neoformans*. Two distinct RAPD fingerprint patterns (designated I to II) were identified by three primers. Three of the 5 strains were assigned to pattern I, and the remaining strains were assigned to pattern II. This result showed the genetic variability of *C. neoformans* serotype A isolates from environmental sources in Busan.

Discussion

C. neoformans serotype A, a major pathogen in immunocompromised patients, is a free living fungus that can be isolated from avian excreta and soils, especially from pigeon droppings. Other environmental isolations have been made from rotting vegetables, wood, and dairy products.

We were successful in recovering *C. neoformans* from environmental sources. Out of the 29 environmental samples, *C. neoformans* was isolated from 5 of 12 samples (41.7%) of pigeon droppings especially from pigeon shelters. The strain was not isolated from soil contaminated avian excreta (0/17). Emmons (1995) established a frequent saprobic association of *C. neoformans* with the old excreta and nests of pigeons. Pal (1997) reported that the prevalence of *C. neoformans* from pigeon droppings was 7 of 28 samples (25%) in Kathmandu. In Thailand, the incidence of *C. neoformans* from chicken feces in suburban areas was 24.0% (Kuroki *et al.*, 2004). Yamamoto *et al.* (1995) reported *C. neoformans* was isolated from 4 of 8 samples (50%) of pigeon excreta in Nagasaki. In the present study, we confirmed the fact that *C. neoformans* var. *grubii* serotype A is highly prevalent in pigeon droppings although limited by the number of environmental sources examined. In our previous report, *C. neoformans* serotype A was the predominant isolate from clinical sources, and *C. neoformans* var. *gattii* serotype B was identified from a patient with meningitis in Korea (Hwang 2002). The studies for the occurrence of environmental sources and clinical disease caused by *C. neoformans* varieties will have some importance in explaining the epidemiological aspects.

Extracellular enzyme activity could be useful methods to help the identification and discrimination of different strains of microorganisms, and to evaluate several putative virulence factors of the strains (Freydiere *et al.*, 1997). According to our results obtained by using the API-ZYM system (Table 3), the most distinctive difference was the production of N-acetyl-β-glucosaminidase (No. 18), which could be helpful to differentiate the phenotypic variation of environmental strains. Leone *et al.* (1998) reported the bird dropping strains were grouped into 14 enzymatic profiles. Absence of alkaline phos-

Table 3. Extracellular enzyme activities of environmental isolates of *C. neoformans*

Strain No.	Strain	Enzyme activity[a]																			
		API ZYM system No.																		PT[b]	PL[c]
		2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	
1	CNS23	+	+	+	–	+	–	–	–	–	+	+	–	–	–	+	+	–	–	–	+	+
2	CNS24	+	+	+	–	+	–	–	–	–	+	+	–	–	–	+	+	+	–	–	+	+
3	CNS25	+	+	+	–	+	–	–	–	–	+	+	–	–	–	+	+	+	–	–	+	+
4	CNS26	+	+	+	–	+	–	–	–	–	+	+	–	–	–	+	+	–	–	–	+	+
5	CNS27	+	+	+	–	+	–	–	–	–	+	+	–	–	–	+	+	–	–	–	+	+

a : +, activity detected; –, no activity detected. b, Protease; c, Phospholipase; API ZYM No. 2, alkaline phosphatase; No. 3, esterase C4; No. 4, esterase lipase C8; No. 5, lipase C14; No. 6, leucine arylamidase; No. 7, valine arylamidase; No. 8, cystine arylamidase; No. 9, trypsin; No. 10, Chymotrypsin; No. 11, acid phosphatase; No. 12, naphthol-AS-BI-phosphohydrolase; No. 13, α-galactosidase; No. 14, β-galactosidase; No. 15, β-glucuronidase; No. 16, α-glucosidase; No. 17, β-glucosidase; No. 18, N-acetyl-β-glucosaminidase; No. 19, α-mannosidase; No. 20, α-fucosidase.

Table 4. RAPD fingerprint patterns of environmental isolates of *C. neoformans*

Strain No.	strain	RAPD profile			Pattern
		Primer OPH-20	Primer OPH-02	Primer R-28	
1	CNS23	A	ii	1	I
2	CNS24	B	i	1	II
3	CNS25	B	i	1	II
4	CNS26	A	ii	1	I
5	CNS27	A	ii	1	I

phatase (No. 2) was a significant difference between their results and ours. Chen *et al.* (1997) has shown that cell associated enzyme assays revealed many additional enzymatic activities.

Cryptococcal extracellular proteins are important because proteinases, esterases, and lipase have been associated with virulence in other pathogens (Tsuboi *et al.*, 1996). Recently, Chen *et al.* (1996) reported that extracellular phospholipase activity produced by *C. neoformans* may disrupt mammalian cell membranes and allow the yeast cells to penetrate into host tissues. Buchanan *et al.* (1998) reported that *C. neoformans* proteases possibly serve as virulence mechanisms by initiating invasion of host tissues. We observed that the activities of these enzymes among all environmental strains and clinical strains (data not shown) showed positive enzymatic activities. This finding has important public health implications.

In general, RAPD analysis offers a sensitive and straightforward system for molecular subtyping of *C. neoformans*. Since it was first described by Williams *et al.* (1990), the suitability of RAPD analysis has been demonstrated by showing its ability to discriminate between closely related isolates within restricted geographic areas (Ruma *et al.*, 1996).

In this study, the interspecies genetic heterogeneity of *C. neoformans* isolates was determined by RAPD by using three 10-mer primers (OPH-20, OPH-02, R-28). With these primers, two major fingerprint patterns (I and II) were identified among the 5 environmental *C. neoformans* isolates from Busan. Our experiment with modified methods of Yamamoto *et al.* (1995) showed a good reproducibility of the RAPD profile. Although our study design does not allow comparison of genetic relatedness among environmental and clinical isolates, it clearly shows that environmental pigeon droppings contained a genetically heterogeneous population of *C. neoformans* serotype A. Also our results revealed a significant correlation between the enzymatic profiles and RAPD patterns.

Currently, further studies of *C. neoformans* isolates from clinical sources are in process to establish the phenotypic and genotypic characterization, and thus obtain a better understanding of the epidemiology of cryptococcosis in Korea.

References

Aoki, S., Ito, K. S., Nakamura, K., Ninomiya, K. and Vitto, V. 1994. Extracellular proteolytic activity of *Cryptococcus neoformans*. *J. Mycopathol.* **128**: 143-150.

Benett, J. E., Kwon-Chung, K. J. and Thodore, T. S. 1978. Biochemical differences between serotypes of *Cryptococcus neoformans*. *Sabouraudia.* **16**: 167-174.

Brandt, M. E., Hutwagner, L. C., Kuykendall, R. J., Pinner, R. W. and The Cryptococcal Disease Active Surveillance Group. 1995. Comparison of multilocus enzyme electrophoresis and random amplified DNA analysis for molecular subtyping of *Cryptococcus neoformans*. *J. Clin. Microbiol.* **33**: 1890-1895.

Buchanan, K. L. and Murphy, J. W. 1998. What makes *Cryptococcus neoformans* a pathogen? *Emer. Infect. Dis.* **4**: 71-83.

Casadevall, A., Freundlich, L. F., Marsh, L. and Scharff, M. D. 1992. Extensive alleric variation in *Cryptococccus neoformans*. *J. Clin. Microbiol.* **30**: 1080-1084.

Chen, L. C., Blank, E. S. and Casadevall, S. 1996. Extracellular proteinase activity of *Cryptococcus neoformans*. *Clin. Diagn. Lab. Immunol.* **3**: 570-574.

_____, Pirofski, L. A. and Casadevall, S. 1997. Extracellular proteins of *Cryptococcus neoformans* and host antibody response. *Infect. Immun.* **65**: 2599-2605.

Currie, B. P. and Casadevall, A. 1994. Estimate of the prevalence of cryptococcal infection among patients infected with the human immunodeficiency virus in New York city. *Clin. Infect. Dis.* **19**: 1029-1033.

Ellis, D. H. 1987. *Cryptococcus neoformans* var. *gattii* in Australia. *J. Clin. Microbiol.* **25**: 430-431.

Emmons, C. W. 1995. Saprophytic sources of *Cryptococcus neoformans* associated with the pigeon (*Columba livia*). *Am. J. Hyg.* **62**: 227-232.

Franzot, S., Salkin, I. F. and Casadevall, A. 1999. *Cryptococcus neoformans* var. *grubii*: Seprate varietal status for *Cryptococcus neoformans* serotype A isolates. *J. Clin. Microbiol.* **37:** 838-840.

Freydiere, A. M. and Guinet, R. 1997. Rapid methods for identification of the most frequent clinical yeasts. *Rev. Iberoam. Micol.* **14**: 85-89.

Hwang, S. M. 2002. Serotyping of *Cryptococcus neoformans* strains isolated in Korea. *J. Microbiol.* **40**: 166-169.

Ikeda, R., Shinoda, T., Fukazawa, Y. and Kaufman, L. 1982. Antigenic characterization of *Cryptococcus neoformans* serotypes and its application to serotyping of clinical isolates. *J. Clin. Microbiol.* **16**: 22-29.

Ito-Kuwa, S., Nagamura, S., Aoki, S., Ninimiya, K., Kato, J. and Vidotto, V. 1994. Serotyping of *Cryptococccus neoformans* isolated from AIDS patients. *Shigaku (Odontology)* **82**: 360-364.

Kuroki, M., Phichaichumpon, C., Yasuoka, A., Chiranairadul, P., Chosa, T., Sirinirund, P., Miyazaki, T., Kakeya, H., Higashiyama, Y., Miyazaki, Y., Ishida, Y. and Kohno, S. 2004. Environmental isolation of *Cryptococcus neoformans* from endemic region of HIV-associated cryptococcosis in Thailand. *Yeast* **21**: 809-812.

Kwon-Chung, K. J., Polacheck, I. and Bennett, J. E. 1982. Improved diagnostic medium for separation of *Cryptococcus neoformans* var. *neoformans* (serotypes A and D) and *Cryptococcus neoformans* var. *gattii* (serotype B and C). *J. Clin.*

Microbiol. **15**: 535-537.

_____ and Bennett, J. E. 1984. Epidemiologic differences between the two varieties of *Cryptococcus neoformans*. *Am. J. Epidemiol.* **120**: 123-130.

Lewis, J. L. and Rabinovich, S. 1972. The wide spectrum of cryptococcal infections. *Am. J. Med.* **53**: 315-322.

Leone, R., Santina, B., Nakamura, K., Aoki, S. and Vidotto, V. 1998. Enzymatic profile of *Cryptococcus neoformans* strains by using the API-ZYM system. *Rev. Ibero. Mycol.* **15**: 136-140.

Meyer, W., Michell, T. G., Freeman, E. and Vilgalys, R. 1993. Hybridization probes for conventional DNA fingerprinting used as single primers in the polymerase chain reaction to distinguish strains of *Cryptococcus neoformans*. *J. Clin. Microbiol.* **31**: 2274-2280.

Pal, M. 1997. First isolation of *Cryptococcus neoformans* var. *neoformans* from avian excreta in Kathumandu, Nepal. *Rev. Iberoam. Micol.* **14**: 181-183.

Pfeiffer, T. J. and Ellis, D. H. 1992. Environmental isolation of *Cryptococcus neoformans* var. *gattii* from Eucalyptus tereticornis. *J. Med. Vet. Mycol.* **30**: 407-408.

Plok, A. 1992. Virulence of *Candida albicans* mutants. *Mycoses* **35**: 9-16.

Ruma, P., Chen, S. C. and Sorrel, T. C. 1996. Characterization of *Cryptococcus neoformans* by random DNA amplification. *Lett. Appl. Microbiol.* 23: 312-316.

Ruiz, A., Fromtling, R. A. and Bulmer, G. S. 1981. Distribution of *Cryptococcus neoformans* in a natural site. *Infect. Immun.* **31**: 560-563.

Sorvillo, F., Beall, G., Turner, P. A., Beer, V. L., Kovacs, A. A. and Kerndt, P. R. 1997. Incidence and factors associated with extrapulmonary cryptococcosis among persons with HIV infection in Los Angeles country. *AIDS* **11**: 673-679.

Tsuboi, R., Komatsuzaki, H. and Ogawa, H. 1996. Induction of an extracellular esterase from *Candida albicans* and some of its properties. *Infect. Immun.* **64**: 2936-2940.

Varma, A., Swinne, D., Staib, F., Bennet, J. E. and Kwon-Chung, K. J. 1995. Diversity of DNA fingerprinting in *Cryptococcus neoformans*. *J. Clin. Microbiol.* **33**: 1807-1814.

Vidotto, V., Sinicco, A., Di Fraia D., Cardaropoil, S. and Ito-Kuwa, S. 1996. Phospholipase activity in *Cryptococcus neoformans*. *Mycopathologia* **136**: 119-123.

_____, Melhim, M., Pukinskas, S., Aoki, S., Carrara, C. and Pugliese, A. 2005. Extracellular enzymatic activity and serotype of *Crytococcus neoformans* strains isolated from AIDS patients in Brazil. *Rev. Iberoam. Micol.* **22**: 29-33.

Walter, J. E. and Coffee, E. G. 1968. Distribution and epidemiologic significance of the serotypes of *Cryptococcus neoformans*. *Am. J. Epidemiol.* **87**: 167-172.

Williams, J. G. K., Kubelik, A. R., Livak, K. J., Rafalski, J. A. and Tingey, A. V. 1990. DNA polymorphism amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res.* **18**: 6532-6535.

Yamamoto, Y., Kohno, S., Koga, H, Kakeya, H., Tomono, K., Kaku, M., Yamazaki, T., Arisawa, M. and Hara, K. 1995. Random amplified polymorphic DNA analysis of clinically and environmentally isolated *Cryptococcus neoformans* in Nagasaki. *J. Clin. Microbiol.* **33**: 3328-3332.

Zhu, H., Feng, Q. and Li-Huang, Z. 1993. Isolation of genomic DNAs from plants, fungi and bacteria using benzyl chloride. *Nucleic Acids Res.* **21**: 5279-5280.