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The isolation of *Cryptococcus neoformans* from pigeon droppings and serotyping of naturally and clinically sourced isolates in China

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Abstract. This is the first report on the isolation of *Cryptococcus neoformans* from pigeon droppings in China and their serotypes. *C. neoformans* colonies which produced brown colonies on caffeic acid-cornmeal agar were found in Twenty-five out of thirty-six samples of pigeon droppings. Fifty-one colonies randomly picked from the positive samples were identified as *C. neoformans* by a commercially available kit for carbon source assimilation test and Christensen's urea agar. Forty (78%) out of the 51 strains were serotyped as A and 11 (22%) as AD. At the same time, seventeen out of nineteen clinical isolates were serotyped as A and 2 as B. There are three findings in our results. One is that only *C. neoformans* var. *neoformans* strains could be isolated from pigeon droppings, although the variety *gattii* strains were found in the clinical isolates obtained in the same geographic site in China. The second is that serotype A strains were most frequently seen in natural and clinical materials in the southeast part of China, and serotype AD strains were isolated in pigeon droppings but not in clinical materials. The third is that the coexistence of serotype A and AD cells of *C. neoformans* strains in same samples of pigeon droppings were observed.

Key words: *Cryptococcus neoformans*, Isolation from environment, Pigeon dropping, Serotyping

Introduction

Cryptococcus neoformans is a clinically important pathogenic fungus. It causes disseminated infection, especially serious meningeal infection in human beings [1]. Because crotypococcosis was more frequently found in patients with immunosuppressive disorders such as AIDS, the significance of research on the ecology of this pathogenic agent has been emphasized [2].

C. neoformans is a yeast like fungus with a capsule and a world-wide distribution. Based on the antigenic determinations of the polysaccharide capsule surrounding cells, *C. neoformans* is subdivided into five serotypes-A, B, C, D and AD [3–5]. These different stereotypes strains were examined biochemically, morphologically and genetically and then designated them as two varieties: *Cryptococcus neoformans* vars. *neoformans* (A/D serogroup) and *Cryptococcus neoformans* var. *gattii* (B/C serogroup) [6, 7]. These serotypes are very useful for research on the etiology, ecology and epidemiology of *C. neoformans* [8–11].

In China, serotypes of clinical isolates of C. neoformans were reported in 1987 [12]. A, AD and B serotypes of *C. neoformans* have been found in patients living in the southeast part of China (Shanghai city and Jiangsu province), but serotypes of natural isolates have never been investigated. We present our results of the isolation of *C. neoformans* strains from pigeon droppings

in Nanjing city, Jiangsu province and the serotypes of the isolates. Some clinical isolates derived from patients in Nanjing city and Shanghai city were used for epidemiological comparison in this study.

Materials and methods

Sabouraud dextrose agar (SDA) (10 g/L polypepton, 20 g/L dextrose, 20 g/L Bact agar) was used to maintain stock cultures and to propagate the tested strains [13]. Caffeic acid cornmeal agar (CACA) slightly modified from Kaufman's methods (0.3 g/L caffeic acid (Sigma, AR), 50 mg/L chloramphenical and 17.5 g/L cornmeal agar (Difco)) [14] was used to isolate *C. neoformans* from pigeon droppings and to detect phenoloxydase activity. Christensen's urea agar (1 g/L polypepton, 1 g/L dextrose, 5 g/L NaCl, 2 g/L KH_2PO_4, 0.012 g/L phenol red, 2 g/L urea and 1.5 g/L agar) was used to examine urease activity [13]. Cornmeal Tween-80 agar was preparated for observation of hyphae produced by tested yeasts [13]. For carbon source assimilation, API 20C SYSTEMS (S.A. Montalieu, France) was used. To group the strains identified as *C. neoformans* into two varieties, canavanine-glycine-bromothymol blue (CGB) medium (0.03 g/L 1-canavanine, 10 g/L glycine, 1 g/L KH_2PO_4,1 g/L $MgSO_4$, 0.001 g/L thiamine, 20 g/L agar and 20 ml/L of 0.4% bromothymol blue) was made by Kwon-Chung's method [7]. Serodiagnostic reagents (Crypto Check) for identification of *C. neoformans* were supplied by Itron Laboratories, Tokyo, Japan. Eight different antisera were contained in a kit and used according to the instructions of the manufacturer.

Clinical isolates. Nineteen strains of *C. neoformans* isolated from spinal fluid of 19 patients with cryptococcosis (none AIDS patients, 5 in Nanjing city and 14 in Shanghai city) were used with 51 avian strains. Five standard strains of *C. neoformans* were IFM 5854 (originally CDC 551, serotype A), IFM 5855 (originally NIH 112, serotype B), IFM 5856 (originally NIH 18, serotype C), IFM 5857 (originally NIH 52, serotype D) and IFM 5889 (serotype AD). (IFM: abbreviation for the Institute of Food Microbiology, former name of Research Center for Pathogenic Fungi and Microbiol Toxicoses, Chiba University, Japan).

Methods. Thirty-six samples of fresh pigeon droppings were collected from three dovecotes of pigeon in Nanjing city during April-June 1990. *C. neoformans* strains were isolated by the following procedures. Briefly, about 1 g samples were mixed with 9 ml sterile saline supplemented with streptomycine and penicillin to the final concentration of 200 μ/ml. After having been vigorously shaken, the suspension was inoculated on CACA medium with a swab. The inoculated plate was incubated at 37 °C for 24 h and then at 25 °C for 6 days. The results were recorded and the brown colonies were randomly picked and inoculated on SDA slants to keep as stock cultures. After two transfers on SDA medium at 25 °C for 72 h, test strains were examined by assimilation of carbon source (API 20C system), tests of urease production and phenoloxydases activities on urea and CACA medium for identification of fungal isolates. Two varieties of *C. neoformans* strains were divided by culture of CGB medium, var. *neoformans* (CGB-negative) and var. *gattii* (CGB-positive). Finally, serotypes of tested strains were determinated by slide agglutination tests (Crypto Check, Iatron Co.). Five standard strains of *C. neoformans* were used as controls in the tests.

Results

Twenty-five random samples of pigeon droppings were found positive for *C. neoformans* in total 36 samples (Table 1). Fifty-one strains which were brown in color on CACA plates were randomly selected into stock cultures and then were confirmed as *C. neoformans* by the tests for identification of fungal isolates. Table 2 shows the results of CGB culture of 70 isolates (51 from avian, 19 from clinical patients). None of the 51 avian

Table 1. Results of isolating *C. neoformans* from pigeon droppings

Devotcotes of pigeons	Number of samples	Isolation +	Isolation −	Number of collected strains
I	12	8	4	20
II	12	7	5	20
III	12	10	2	11
Total	36	25	11	51

Table 2. Serogrouping results of *C. neoformans* strains with the method of culture on CGB medium

Origin	Number	CGB +	CGB −	CACA +	CACA −	Urea agar +	Urea agar −
Clinical							
Shanghai	14	2	12	14	0	14	0
Nanjing	5	0	5	5	0	5	0
Avian	51	0	51	51	0	50	1
Standard							
Group I[a]	3	0	3	3	0	3	0
Group II[b]	2	2	0	2	0	2	0

[a] Var. *neoformans* strains including IFM 5854, IFM 5857 and IFM 5889.
[b] Var. *gattii* strains including IFM 5855 and IFM 5856.

isolates were on CGB medium. Two out of 19 clinical isolates were positive on CGB medium. Table 2 also shows the results of culture on CACA medium and urea agar respectively. All strains tested had a positive reaction for phenoloxydase on CACA medium. Except for one strain, fifty avian isolates were positive reaction of urease on urea agar. This urease negative strain of *C. neoformans* found in pigeon dropping has been reported previously [25].

Table 3 shows serotypes of avian and clinical isolates. Seventy-tested strains were serotyped by factor sera for *C. neoformans* (Crypto Check, Iatron Co.). Eleven out of 51 avian isolates were serotyped as AD (22%) and 40 (78%) as A, no B or C serotypes were found. Those eleven AD serotype strains were smeared again on CACA plates and then eleven single colones were repeatedly serotyped to exclude the possible mixture of serotype A and AD. Those two strains of serotype B were from patients in Shanghai city. Five strains from patients in Nanjing city were serotype A.

The distribution of A and AD serotypes of *C. neoformans* in pigeon droppings was analyzed according to the serotyping results. In 25 positive samples of pigeon droppings (reference to Table 1), six samples contained both A and AD serotype. In other 19 samples, seventeen contained only serotype A and two contained serotype AD (data are not shown in Table 4).

Discussion

It is well known that *C. neoformans* can be commonly isolated from pigeon droppings or soil enriched with pigeon excreta [15–17]. Pigeons are considered to be a carrier of *C. neoformans* and to play an important role in the dissemination of this pathogenic fungus [18, 19]. As shown in Table 1, 69% positive isolation rate was found in pigeon droppings in China. This suggests that the natural habitant of *C. neoformans* in China is similar to previously reported findings in other countries.

Even though *C. neoformans* can be subdivided into five serotypes or two varieties, until now only A/D serogroup (var. *neoformans*) strains have been isolated from pigeon droppings, even in some area where B/C serogroup (var. *gattii*) are prevalent in clinical patients [8, 9, 20]. In 1987, it was reported that five serotype B strains of *C. neoformans* were isolated from clinical patients in Shanghai city and Jiangsu province [12]. In this report, we also found two serotype B strains from clinical isolates (Table 3). In fact, Shanghai city and Nanjing city are two close cities in the southeast part of China and enjoy a subtropical climate. Therefore we suggest that serotype B (var. *gattii*) strains are endemic in the southeast part of China. On the other hand, we failed to isolate serotype B or C (var. *gattii*) strains from pigeon droppings (Table 3). These results suggest that var. *gattii* may be absent in pigeon droppings in China, although *C. neoformans* is isolated frequently from pigeon droppings and var. *gattii* could be isolated from clinical patients in the correlated geographical site of China.

Recently, it has been implicated by accumu-

Table 3. Serotyping results of *C. neoformans* strains by the slide agglutination method

Origin	Number of isolates	Slide agglutination with factor antisera								Serotype
		1	2	3	4	5	6	7	8	
Clinical										
Shanghai	2	+	+	−	+	+	−	−	−	B
	12	+	+	+	−	−	−	+	−	A
Nanjing	5	+	+	+	−	−	−	+	−	A
Avian	11	+	+	+	−	−	−	+	+	AD
	40	+	+	+	−	−	−	+	−	A
Standard										
IFM 5854	1	+	+	+	−	−	−	+	−	A
IFM 5855	1	+	+	−	+	+	−	−	−	B
IFM 5856	1	+	−	−	+	−	+	−	−	C
IFM 5957	1	+	+	+	−	−	−	−	+	D
IFM 5889	1	+	+	+	−	−	−	+	+	AD

Table 4. Coexisting of serotype A and AD of *C. neoformans* strains in one pigeon dropping

Sample	Number of isolated strains	Serotyping results	
		A	AD
I-5	7	6	1
I-6	2	1	1
I-10	2	1	1
II-2	4	3	1
II-5	3	2	1
II-8	3	2	1

lated data, that the two varieties of *C. neoformans* may have different natural habitats. The habitat of *C. neoformans* var. *gattii* was correlated with a host tree- *Eucalyptus camaldulenesis* according to Ellis and Pfeiffer [21–23]. In 1992, Kwon-Chung confirmed that *Eucalyptus*-originated *C. neoformans* var. *gattii* was the same organism as those isolated from cases of human infections by studies on virulence, serotyping and analysis of electrophoretic karyotypes [24]. Therefore, it is interesting to compare the pattern of serotypes in var. *neoformans* strains isolated from pigeon droppings and those isolated from clinical patients. Even though all var. *neoformans* isolates from clinical patients were serotyped as A in this study, serotype A and AD strains of var. *neoformans* from clinical isolates were previously reported in Jiangsu and Shanghai [12]. In that report, 35 isolates of var. *neoformans* from clinical patients in Jiangsu and Shanghai area were serotyped as 32 (92%) A and 3 (8%) AD. In this study, 51 isolates of var. *neoformans* derived from pigeon droppings were serotyped into 22% AD and 78% A (Table 3). If these two grougs of data are compared, it will be found that the serotype patterns of var. *neoformans* isolates from clinical source and pigeon droppings are similar. Because serotype A and AD strains of var. *neoformans* were commonly seen in the materials from those two sources in the southeast part of China, it is suggested that var. *neoformans* strains in pigeon droppings are the important sources of clinical infections.

In Table 4, we present interesting ecological information of *C. neoformans* var. *neoformans*. Two serotypes of var. *neoformans* (A and AD) strains were found coexisting in one sample of pigeon dropping. We believe this phenomenon has not been reported previously. The significance of this finding still needs to be clarified. However, a urease negative strain of *C. neoformans* (serotype A) has been found in our early researchs [25]. It was isolated from pigeon droppings collected in dovecote III shown in Table 1 and Table 2. Another urease negative strain of *C. neoformans* (serotype A) was isolated by Ruane et al. from an AIDS patients in the USA [26]. According to these findings, we probably propose that the phenotype of var. *neoformans* is

highly polymorphic in its natural habits and this polymorphism of the organism causes a biodiversity of clinical isolates.

References

1. Rippon JW. Medical mycology: The pathogenic fungi and pathoenic actinomycetes, 2nd. ed. Philadelphia: W.B. Saunders, 1982: 532–58.
2. Sugar AM. Overview: *Cryptococcus neoformans* in the patient with AIDS. Mycopathologia 1991; 114(3): 153–57.
3. Evans EE. The antigenic composition of *Cryptococcus neoformans*, I: Serologic classification by means of the capsular and agglutation reactions. J Immunol 1951; 67: 109–14.
4. Wilson DE, Bennett JE, Bailley JW. Serologic grouping of *Cryptococcus neoformans*. Proc Soc Exp Biol Med 1968; 127: 820–23.
5. Ikeda R, Shinoda T, Fukazawa Y, Kaufman L. Antigenic characterization of *Cryptococcus neoformans* serotypes and its application to serotyping of clinical isolates. J Clin Microbiol 1982; 16(1): 22–29.
6. Kwon-Chung KJ, Bennet JE, Theodore TS. *Cryptococcus bacillisporus* sp. nov serotype B-C of *Cryptococcus neoformans*. Int J System Bact 1978; 28: 616–20.
7. Kwon-Chung KJ, Polacheck I, Bennett JE. Improved diagnostic medium for separation of *Cryptococcus neoformans* var. *neoformans* (serotype A and D) and *Cryptococcus neoformans* var. *gattii* (serotype B and C). J Clin Microbiol 1982; 15(2): 535–37.
8. Bennett JE, Kwon-Chung KJ, Dexter-Howard. Epidermiologic differences among serotypes of *Cryptococcus neoformans*. Am J Epide 1977; 195(6): 582–86.
9. Kwon-Chung KJ, Bennett JE. High prevalence of *Cryptococcus neoformans* var. *gattii* in tropical and subtropical regions. Zbl Bakt Hyg 1984; A257: 213–18.
10. Shadomy HJ, Wood-Helie S, Shadomy S, Dismukes WE, Chau RY, NIAID Mycoses Study Group. Biochemical serogrouping of clinical isolates of *Cryptococcus neoformans*. Dian Microbiol Infect Dis 1987; 6: 131–38.
11. Rozenbaum R, Rios-Goncalves AJ, Wanke B, Cainby MJ, Clemente H, Santos-Lazera MD, Fialho-Monteiro, Londero AT. *Cryptococcus neoformans* varieties as agents of cryptococcosis in Brazil. Mycopathologia 1992; 119: 133–36.
12. Li ZQ, Shao JZ, Liao WQ, Li SQ, Kong XT, Shie YH. A study on the serotypes of pathogenic *Cryptococcus neoformans* strains in China. Chin J Med Lab Tech 1987; 10(6): 350–52.
13. Moore GS, Jaciow DM. Mycology for the Clinical Laboratory. Reston, VA: Reston Publishing Co, 1979: 283–300.
14. Kaufmann CS, Merz WG. Two rapid pigmentation tests for identification of *Cryptococcus neoformans*. J Clin Microbiol 1982; 15(2): 339–41.
15. Bulmer GS. Twenty-five years with *Cryptococcus neoformans*. Mycopathologia 1990; 109: 111–22.
16. Ruiz A, Velez D, Fromtling RA. Isolation of *Cryptococcus neoformans* from Puertio Rico: Distribution and variety. Mycopathologia 1988; 106(3): 167–70.
17. Swinne D, Deppner M, Maniratunga S, Laroche R, Floch JJ, Kadende P. AIDS-associated cryptococosis in Bujenbura, Burundi: An epidemiological study. J Med and Veter Mycolo 1991; 29: 25–30.
18. Swinne-Desgain D. *Cryptococcus neoformans* in the crops of pigeons following its experimental administration. Sabouraudia 1976; 14: 313–57.
19. Abou-Gabal M and Atia M. Study of the role of pigeons in the dissemination of *Cryptococcus neoformans* in nature. Sabouraudia 1978; 16: 63–68.
20. Swinne D. *Cryptococcus neoformans* and the epidemiology of cryptococcosis. Ann Sco Belgium Med Trop 1979; 59: 285–99.
21. Ellis DH, Pfeiffer TJ. Natural habital of *Cryptococcus neoformans* var. *gattii*. J Clin Microbiol 1990; 28(7): 1642–44.
22. Pfeiffer TJ, Ellis DH. Environmental isolation of *Cryptococcus neoformans* var. *gattii* from California. JID 1991; 163: 929–930.
23. Ellis DH, Pfeiffer TJ. Life cycle and infectious propagule of Cryptococcus neoformans. Lancet 1990; 336: 923–25.
24. Kwon-Chunh KJ, Wickes BL, Stockman L, Robèter GD, Ellis DH, Howard DH. Virulence, serotype and molecular characteristics of environmental strains of *Cryptococcus neoformans* var. *gatii*. Infect and Immun 1992; 60(5): 1869–82.
25. Li AS, Wu SX. An urease negative *Cryptococcus neoformans* strain. Acta Microbiol Sinica 1992; 32(1): 68–71.
26. Ruane PJ, Walker LJ, George WL. Disseminated infection caused by urease-negative *Cryptococcus neofornans*. J Clin Microbiol 1988; 26(10): 2224–25.

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