

Epidemiology Studies of Clinical Isolates of *Cryptococcus neoformans* of Japan by Restriction Fragment Length Polymorphism

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Abstract

Previous epidemiological studies of *Cryptococcus neoformans* infection in Japan showed that only *C. neoformans* var. *neoformans* is present and serotype A is the most common with frequencies in excess of 95%. A DNA fingerprinting method, using a genomic DNA probe (UT-4p), has become available recently which discriminates between individual isolates in a population that are morphologically and serologically indistinguishable. Fifty-two serotype A isolates of *C. neoformans* were obtained from three different institutions (in Nagasaki, Chiba, and Tokyo) in Japan. Only two of these strains were isolated from AIDS patients and one from pigeon excreta. Of the nine reported fingerprinting patterns in serotype A, only three types (IV, V and VII) were observed in Japanese isolates. Pattern IV was almost exclusively observed in Nagasaki isolates (21/22) with only one of pattern VII. In Chiba, however, patterns VII and IV appeared to be equally distributed. In Tokyo, patterns IV and V (which included two isolates from AIDS patients) were observed at similar frequencies.

Restriction fragment length polymorphism analysis of four isolates of serotype AD showed a typical serotype A pattern which also contained a serotype D-specific band. This finding suggests the independence of serotype AD. These data could enhance the survey of the epidemiology of cryptococcosis.

Introduction

In Japan, cryptococcosis is the third most common fungal infection among deep-seated mycoses. Its exact occurrence rate is not known; however, pathological examination revealed cryptococcosis in about 0.3-0.5% of all autopsied cases¹⁾. Cryptococcosis occurs sporadically, but it has increased sharply in Western Europe and the United States because of the increase in acquired immunodeficiency syndrome (AIDS). The total number of AIDS patients was 764 and that of human immunodeficiency virus-infected persons was 3389 by the end of June 1994 in Japan. The prevalence of cryptococcosis among AIDS patients appears to be approximately 3-8%²⁾, which suggests that not many cases of

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cryptococcosis could occur among AIDS patients in Japan. The epidemiology of cryptococcosis shows that AIDS patients were almost always infected with serotype A isolates of the variety *neoformans*³⁾. Previous epidemiological studies of the disease in Japan showed that only *C. neoformans* var. *neoformans* is present and serotype A is the most common, with frequencies in excess of 95%.

In order to discriminate between individual isolates in a population that are morphologically and physiologically indistinguishable, a stable and sensitive genetic marker is needed. The DNA fingerprinting method, which involves highly variable restriction fragment length polymorphism (RFLP), was used to investigate the epidemiology of cryptococcosis in Japan, using a genomic DNA probe (UT-4p).

The anamorph of serotypes A and D is *C. neoformans* var. *neoformans* and that of serotypes B and C is *C. neoformans* var. *gattii*. The anamorph of serotype AD is still controversial. The RFLP analysis was performed to investigate whether serotype AD of *C. neoformans* has a characteristic DNA pattern.

Materials and Methods

Clinical and environmental isolates

Twenty-one clinical isolates of *C. neoformans* var. *neoformans* serotype A from non-AIDS patients with cryptococcosis and one environmental isolate from pigeon excreta were obtained from Nagasaki University Hospital and related hospitals in Nagasaki prefecture (Table 1). Eighteen clinical isolates (IFM 5807, IFM 5809-5810, IFM 5812-5814, IFM 5820-5821, IFM 5823, IFM 5826-5832, IFM 5836-5837) from non-AIDS patients were kindly provided by Prof. K. Nishimura at the Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University; 10 clinical isolates (9001, 9002, 9006, 9007, 9011, 9013, G 1345, G 2718, 356, 506) from non-AIDS patients and two clinical isolates

Table 1 Clinical isolates of *Cryptococcus neoformans* var. *neoformans* in Nagasaki

Strain	Sample	Isolated place
NACR 4	Percutaneous lung aspirate	Nagasaki
NACR 12	Pigeon excreta	Nagasaki
NACR 13	Autopsied lung	Nagasaki
NACR 19	Transbronchial aspirate	Nagasaki
NACR 21	Percutaneous lung aspirate	Nagasaki
NACR 22	Sputum	Nagasaki
NACR 30	Sputum	Nagasaki
NACR 31	Transbronchial lung biopsy	Nagasaki
NACR 32	Percutaneous lung aspirate	Nagasaki
NACR 26	Sputum	Nagasaki
NACR 27	Cerebrospinal fluid	Nagasaki
NACR 11	Blood	Nagasaki
NACR 18	Cerebrospinal fluid	Hokusho
NACR 23	Cerebrospinal fluid	Nagasaki
NACR 29	Bronchoalveolar lavage	Nagasaki
NACR 14	Cerebrospinal fluid	Isahaya
NACR 28	Bronchoalveolar lavage	Isahaya
NACR 15	Transbronchial lung biopsy	Sasebo
NACR 16	Sputum	Sasebo
NACR 17	Transbronchial aspirate	Sasebo
NACR 24	Transbronchial lung biopsy	Hokusho
NACR 25	Cerebrospinal fluid	Tarami

(608, 808) from AIDS patients by Prof. K. Shimada at the Institute of Medical Science, the University of Tokyo; and 4 isolates (R 809-812) of *C. neoformans* var. *neoformans* serotype AD by Prof. T. Shinoda at Meiji College of Pharmacy, Tokyo. All isolates were confirmed as *C. neoformans* var. *neoformans* based on the reaction on canavanine-glycine-bromothymol blue medium and serotypes were confirmed by Crypto Check® (Iatron, Tokyo, Japan).

RFLP analysis

The cultures were maintained on YEPD (1% yeast extract, 2% Bacto-Peptone, and 2% glucose) agar slants, and log-phase cells cultured on the agar containing 1% yeast extract and 2% glucose at 30°C were harvested. Genomic DNA was extracted from each isolate as previously described⁴⁾. Briefly, protoplasts were formed by treatment with mureinase (5 mg/ml; United States Biochemical

Fig. 1 DNA fingerprinting patterns of serotype A isolates of *Cryptococcus neoformans* var. *neoformans*

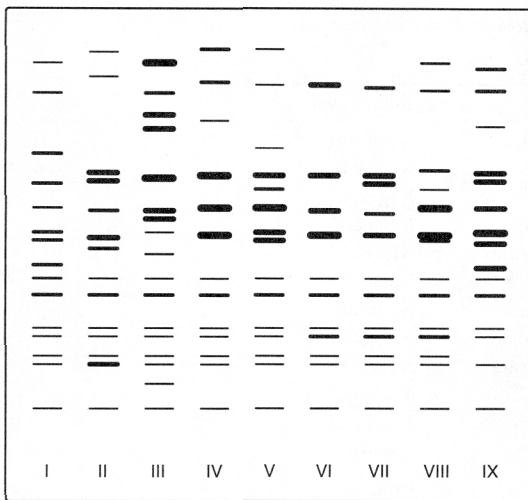
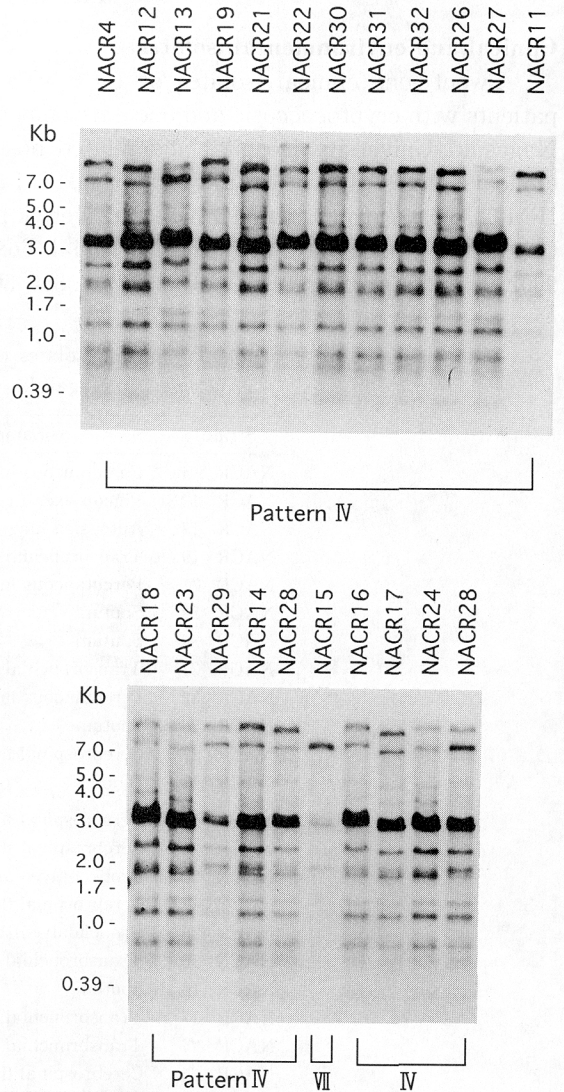


Fig. 2 Southern blot autoradiograms of Nagasaki isolates illustrating RFLP of ACC1-digested DNA probed with UT-4p



Corp.) in a wash solution containing 20 mmol of sodium citrate (pH 5.8) and 1 mol of sorbitol. Cells were lysed with a solution containing 0.1 mol of EDTA (pH 8), 10 mmol of Tris-HCl (pH 8), 1% sarkosyl, and 1 mg of proteinase K (Boehringer, Mannheim, Germany) per ml. After treatment with RNase (10 mg/ml; Sigma Chemical Co. St. Louis, Mo.) and proteinase K (20 mg/ml), phenol (Bethesda Research Laboratories, Gaithersburg, Md.) and chloroform (J.T. Baker, Phillipsburg, N.J.) extraction was performed. Between 3 and 5 μ g of genomic DNA extracted from each of the selected isolates was electrophoresed on 0.8% agarose after being digested with the restriction endonuclease Acc 1.⁵⁾ The DNA was then transferred to Nytran filters (Schleicher & Schuell) and they were hybridized with the UT-4p probe labelled with [³²P] dCTP (Amersham, Oak Park, Ill.).

Results

Acc1-digested DNA probed with UT-4p from the Japanese isolates revealed only three patterns (IV, V, VII) of RFLP, which gave nine patterns according to A. Varma (Fig. 1) (A. Varma et al., personal communication). Pattern IV was predominant among the Nagasaki isolates (21/22), which included one environmental isolate. The only exception was pattern VII of strain NACR 15, which was isolated from a 49-year-old man with pulmonary cryptococcosis (Fig. 2). RFLP of Chiba isolates showed that there were three patterns (IV, V, VII) among which pattern IV and VII appeared to be equally distributed (Fig. 3). In Tokyo, isolates of pattern IV and V (which included 2 AIDS isolates) were observed at similar frequencies (Fig. 3). Table 2 summarizes the RFLP patterns for all isolates.

Fig. 3 Southern blot autoradiograms of Chiba and Tokyo isolates illustrating RFLP of ACC1-digested DNA probed with UT-4p.

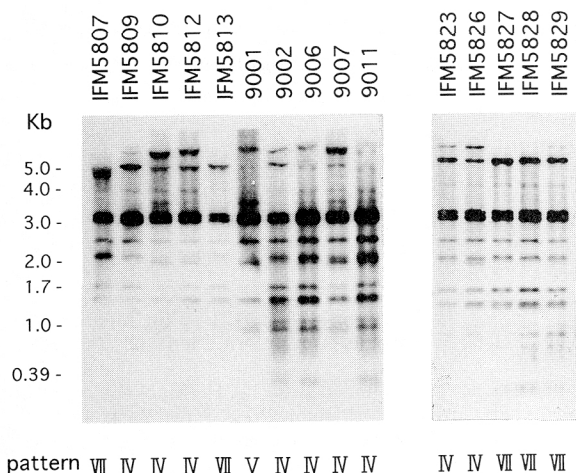


Fig. 4 Southern blot autoradiograms of serotypes AD isolates of *Cryptococcus neoformans* var. *neoformans* illustrating RFLP of ACC1-digested DNA probed with UT-4p.

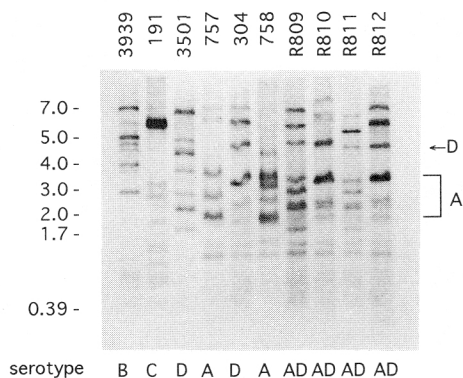


Table 2 The patterns of RFLP and the geographic sources of *Cryptococcus neoformans* var. *neoformans* serotype A in Japan

Pattern	No. of isolates	Nagasaki		Chiba	Tokyo	
		Non-AIDS	Environment	Non-AIDS	AIDS	Non-AIDS
IV	36	20	1	8		7
V	7			2	2	3
VII	9	1		8		
Total	52	21	1	18	2	10

RFLP analysis of four isolates of serotype AD showed a typical serotype A pattern which also contained a serotype D-specific band (Fig. 4).

Discussion

Several classification methods have been used in epidemiological studies including serotyping⁶⁾, electrophoretic karyotyping⁷⁾, use of mitochondrial DNA probes⁸⁾, use of genomic DNA probes^{5,9)}, and allelic variations at the *URA5* locus¹⁰⁾. They all are useful for differentiating isolates to some extent; however, they do not provide any significant information on population composition. DNA fingerprinting methods, which involve highly variable RFLP, have the ability to discriminate between individual isolates in a given population. Varma determined the DNA fingerprinting patterns of serotype A isolates of *C. neoformans* from California, Berlin, and Zaire, and strains at the U.S. National Institutes of Health by using a genomic probe (UT-4p) (A. Varma et al., personal communication). Patterns II and V were consistently observed in patients from most geographic areas with pattern V being more abundant than pattern II. Patterns IV and VII were only seen in patients from southern California; however, they are very common in Japan. Pattern IV was almost exclusively observed in Nagasaki isolates with only one of pattern VII. In Chiba, however, patterns VII and IV appeared to be equally distributed. In Tokyo, patterns IV and V (which included 2 AIDS isolates) were observed at similar frequencies. These patterns are characteristic of not only worldwide distribution but also domestic distribution.

The serotype has been useful for the ecological and epidemiological differentiation between the two varieties, *C. neoformans* var. *neoformans* and var. *gattii*. Serotype AD is still controversial regarding its independence. We examined the RFLP patterns of serotype AD to attempt to determine its independence, and the results suggest that serotype AD is independent.

Our results are preliminary, and they must be considered within the limitations imposed by small sample sizes. Continued research will result in large sample sizes, better recognition of the genetic variability of clinical and environmental isolates, and the status of serotype AD.

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本邦における *Cryptococcus neoformans* 臨床分離株の疫学的検討¹⁾長崎大学医学部第二内科, ²⁾LCI, NIAID河野 茂¹⁾ Ashok VARMA²⁾ K.J. KWON-CHUNG²⁾ 原 耕平¹⁾

要 旨

本邦における *Cryptococcus neoformans* の臨床分離株は *C. neoformans* var. *neoformans* であり, 約95%は血清型 A である。我々は, UT-4p をプローブとして, ACC1で処理した *C. neoformans* の DNA を DNA fingerprinting 法で疫学的に検討した。長崎, 千葉, 東京で分離された血清型 A の計52株の RFLP パターンは特徴的型を示した。世界的には9パターンが知られ, 最も多いパターンはVであったが, 長崎では, 1株(VII)

を除き, 全てパターン IV (鳩の糞からの分離1株も含めて) であった。千葉では, パターン IV と VII がほぼ同じ頻度で, 東京ではパターン IV と V (AIDS 患者からの2株を含めて) がみられた。なお, パターン IV と VII (1株のみ) は California でしか分離されていない。

血清型 AD の独立性は, 未だ一般には認められていないが, 4株の RFLP パターンでは, 血清型 A に典型的なパターンに血清型 D 特異的バンドがみられ, その独立性が強く示唆された。