Postgrad Med J 2000;**76**:85–88 © The Fellowship of Postgraduate Medicine, 2000

Original articles

Cryptococcosis in AIDS

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Summary

A total of 87 patients (17 female, 70 male) were admitted to Siriraj Hospital, Mahidol University, Bangkok, Thailand, from January 1996 to December 1997, with a diagnosis of cryptococcal meningitis and underlying AIDS. The age range was 14–70 years, mean 32.1. Six females (35%) and thirty-one males (44%) died, while the others were discharged home after clinical improvement. The mean duration of admission of those who died was 14.5 days, which was shorter than that of the patients who survived (25.7 days). Cerebral cryptococcosis was diagnosed using culture (100%), India ink preparation (91%), latex agglutination test (100%), and polymerase chain reaction (86%). Polymerase chain reaction fingerprinting of *Cryptococcus neoformans* revealed 99% serotype A and 1% serotype B. The mean minimum inhibitory concentrations of amphotericin B, flucytosine, fluconazole and itraconazole against 87 isolates of *C neoformans* were 0.55 µg/ml (0.25–1, SD = 0.22), 9.5 µg/ml (2–20, SD = 4.91), 6.9 µg/ml (1–16, SD = 4.42) and 0.36 µg/ml (0.125–1.0, SD = 0.23), respectively. These findings showed that the cryptococcal infections were sensitive to these antifungal agents.

Keywords : *Cryptococcus neoformans;* AIDS; polymerase chain reaction fingerprinting; antifungal agents

Fungal infections are an important problem in medicine nowadays. The rate of fungal infection in Thailand is increasing, not only because of environmental factors that support the growth of several fungi, but also because there are an increasing number of immunocompromised patients, particularly those with AIDS. *Cryptococcus neoformans,* the organism causing cryptococcosis, was first reported to be pathogenic to man in 1894 and was isolated from pigeon manure in 1955.[1] The first reported case of cryptococcosis in Thailand was recorded in 1960.[2] This pathogenic organism is known as an encapsulated yeast and has a worldwide distribution. Among AIDS patients in Thailand, cryptococcosis is the second most common opportunistic infection after tuberculosis and most patients have primary systemic fungal infections and late HIV disease.

The identification of this fungus is based on its morphological and physiological characteristics. Polymerase chain reaction (PCR) with specific primers has been introduced for diagnosing cryptococcosis.[3] Moreover, two genetically distinct varieties of *C neoformans* are recognised: *C neoformans* var *neoformans* and *C neoformans* var *gattii.*[4] The varieties can be distinguished by their differences in growth on diagnostic media[5] and by testing with monoclonal antibodies specific for each type.[6] Recently, PCR fingerprinting has been introduced[7]; this method has been reported as being stable.[8] The purpose of this study was to investigate the presentation, underlying diseases, outcome of infected patients, serotype and drug susceptibility of *C neoformans* isolated from patients admitted to Siriraj Hospital, Mahidol University, Bangkok, Thailand, from January 1996 to December 1997. Furthermore, we wished to compare conventional and PCR methods for diagnosing cryptococcosis.

Materials and methods

SPECIMENS

A total of 87 isolates of *C neoformans* were cultured from the cerebrospinal fluid (CSF) of 17 female and 70 male patients admitted to Siriraj Hospital between January 1996 and December 1997. The culture medium used was Sabouraud dextrose agar (SDA) without cycloheximide. Identification of *C neoformans* was based on India ink preparation, growth in culture at 37°C, a positive urease test, and a positive phenoloxidase test.

LATEX AGGLUTINATION TEST

A slide latex agglutination test for cryptococcal antigen with titres for each specimen was performed using a locally made LA-kit (Center for Immunodiagnostic Production, Mahidol University). This preparation was polystyrene latex particles coated with *C neoformans* antibody. The specimens were first inactivated at 56°C for 30 min, and then tested with anti-cryptococcal globulin latex reagent in the presence of glycine buffer, and rotated for 5 minutes at room temperature. The titre was determined by two-fold serial dilution.

PREPARATION OF DNA FROM CLINICAL SPECIMENS

A 1-ml sample of CSF was centrifuged at 12 000 rpm for 5 min to collect cells; the pellet was then resuspended in 0.5 ml of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). The cell suspension was transferred to a 1.5 ml microcentrifuge tube which contained 0.5 ml of sterile siliconised glass beads (ϕ 425–600 µm; Sigma, USA). The cells were broken by vortexing on the mixer for 1 min and alternately placing on ice for 1 min. The procedure was repeated 10 times. Then the mixture was heated in a boiling water bath for 10 min. The mixture was centri-

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Submitted 12 February 1999
Accepted 7 June 1999

fuged at 12 000 rpm for 5 min and the supernatant was removed to a new tube. DNA in the supernatant was precipitated as previously described by Bowman[9], resuspended in 10 µl of TE (10:1) and stored at -20°C until used.

PCR PRIMERS FOR *C neoformans*

The forward primer was CPL1 (5'-GGAGGTAGTGACAATAAATA-3', nucleotide 459–478) and the reverse primer CPR 4 (5'-TGCTAATGTATTCGGGCGATT-3', nucleotide 801–781). These primers were expected to amplify a fragment of 343 bp within the 18 S rDNA of *C neoformans.*[3] The primers were synthesised by the Bio-Service Unit, Mahidol University.

DNA AMPLIFICATION

The polymerase chain reaction was carried out in 600 µl microcentrifuge tube (Robbin, USA) containing 50 mM KCl, 10 mM Tris (pH 8.4), 2.5 mM $MgCl_2$, 0.01% gelatin, 25 pmol of each primer, 200 µmol of each deoxynucleotide triphosphate and 1 unit of *Taq* polymerase (BRL, USA). PCR was performed in a DNA thermal cycler (Perkin-Elmer Cetus 480, USA) with denaturation at 94°C for 5 min, followed by 50 cycles of denaturation at 94°C for 45 s, annealing at 62°C for 45 s and extension at 72°C for 45 s. There was one reagent control, one negative control and two positive control tubes included in each set of PCR. The amplification results were analysed only when all the controls produced the correct results.

AGAROSE GEL ELECTROPHORESIS

Samples of 6 µl from each PCR reaction tube were electrophoresed in 4% Nusieve-agarose (FMC, Germany) and the amplified DNA band was stained with ethidium bromide. Analysis of the amplification was accomplished by taking photographs of gels on a UV-transilluminator (Spectroline® TVC-312 A, USA) with a Polaroid camera (Fotodyne, USA) and then taking note of negative and positive DNA bands in each well.

DNA ISOLATION FOR PCR FINGERPRINTING

DNA was isolated by the physical rupture technique which used glass beads to break cells for isolation of DNA. This method was performed in 1.5-ml microcentrifuge tubes. Yeast cells from SDA were scraped and resuspended into 500 µl of TE buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA). Cells were washed once with 500 µl of TE buffer and centrifuged at 12 000 rpm for 2 min. The pellet was resuspended in 500 µl of TE buffer and the suspension was transferred to a new microcentrifuge tube containing 0.5 ml of glass beads (ϕ 425–600 µm; Sigma, USA). Cells were broken by vortexing the tube for 1 min and immediately placing it on ice for 1 min. This step was repeated five times. To the suspension was added 1/3 volume of lysis buffer (50 mM Tris-HCl pH 7.2, 50 mM EDTA, 1% β-mercaptoethanol and 3% sodium dodecyl sulfate) and it was mixed by inversion. The mixture was incubated at 65°C for 1 h and then centrifuged at 12 000 rpm for 2 min. The supernatant was transferred to a new microcentrifuge tube, and extracted once with phenol, once with phenol:chloroform:isoamyl alcohol (25:24:1), and finally with chloroform-:isoamyl alcohol. DNA was precipitated with ethanol, washed with 70% ethanol, dried and resuspended in 50 µl of TE buffer. Finally, 5 µl of ribonuclease A was added and the DNA incubated at 37° C for 1 h. The purified DNA was stored at -20°C.

PRIMERS FOR PCR FINGERPRINTING

The phage M13 core sequence 5'-GAGGGTGGXGGXTCT-3' was used for PCR to amplify hypervariable and repetitive sequences of *C neoformans.*[7] The primer was synthesised by Bio-Service Unit, Mahidol University.

PCR FINGERPRINTING

PCR fingerprinting was established by using prototype strains of *C neoformans* serotypes A, B, C, and D. The PCR fingerprinting procedure was performed in 0.6-ml microcentrifuge tubes each with a total volume of 50 µl, containing 10–25 ng of genomic DNA, 0.2 mM deoxynucleotide triphosphate (dNTP), 1.5 mM magnesium chloride, 30 ng primer and 2.5 unit of *Taq* DNA polymerase. Under the recommended buffer conditions, the PCR was performed for 40 cycles in a DNA thermal cycler (Perkin-Elmer Cetus, 480, USA) as follows: denaturation at 93°C for 20 s, annealing at 50°C for 60 s, extension at 72°C for 20 s, followed by a final extension step at 72°C for 6 min. The amplification products were analysed by 4% Nuseive-Agarose gel electrophoresis run in Tris acetate buffer, and detected by staining with ethidium bromide and viewed on a UV transilluminator.

DRUG SUSCEPTIBILITY

Drug susceptibility tests were performed by the macrodilution method using yeast nitrogen base media. Four antifungal agents were used: amphotericin B (Squibb, Princeton, NJ), flucytosine (Hoffmann La Roche, Inc, Nutley, NJ), fluconazole (Pfizer, Sandwich, UK) and itraconazole (Janssen Pharmaceuticals, Piscataway, NJ). Final drug concentrations ranged from 32 to 0.015 µg/ml by two-fold serial dilution. The yeast cells were prepared from a 48-h culture on SDA tubes incubated at 35° C, then suspended in sterile 0.85% normal saline solution to obtain a final dilution of $1–5 \times 10^5$ cells/ml; 0.1 ml was added to each tube. Minimum inhibitory concentrations (MIC) were read at 24 and 48 h using end-point criteria as defined by the National Committee for Clinical Laboratory Standards.[10]

Results

Isolates of *C neoformans* were cultured from each of 87 patients (17 females, 70 males) with cryptococcal meningitis. The age ranges were: females 17–70 years (mean 30.8, SD 13.8); males 14–60 years (mean 32.4, SD 8.5). HIV infection was the underlying disease in all patients. Risk exposure for HIV infection was

Table Sex, age, CD4 lymphocyte count, and duration of hospitalisation of cryptococcal meningitis patients with AIDS

	Females (n = 17)	*Males (n = 70)*	*Total (n = 87)*
Age (years)*	30.8 ± 13.8	32.4 ± 8.5	32.1 ± 9.6
range	17–70	14–60	14–70
CD4 lymphocyte count (/mm^3)	59	41	45
range	9–205	4–212	4–212
Length of hospitalisation (days)			
patients who survived (range)	23 (14–31)	26 (5–58)	25 (5–58)
patients who died (range)	24 (1–79)	12 (2–36)	14 (1–79)

*Values are mean ± SD

heterosexual contact for all except one (the 14-year-old boy). CD4 lymphocyte counts for female and male patients ranged from 9–205 mm^3 (mean 59.0, SD 73.4) and 4–212 mm^3 (mean 41.8, SD 50.8), respectively. Eleven female and 39 male patients were discharged with clinical improvement. The duration of admission was 14–31 days (mean 23.4, SD 6.4) in females and 5–58 days (mean 26.2, SD 14.2) in males. Six female and 31 male patients died in hospital, survival after admission ranging from 1–79 days (mean 24.4, SD 33.6) in females, and 2–36 days (mean 12.3, SD 11.8) in males (table).

The median cryptococcal antigen titre of the initial CSF latex agglutination test was 1:16 (range 1:4–1:128). After 3 weeks of treatment, CSF revealed no growth in 80% of patients, but the latex agglutination test for cryptococcal antigen titre remained positive.

Using culture as the gold standard, the sensitivities of the other methods for diagnosing cryptococcosis were: India ink preparation 91%, latex agglutination 100%, and PCR 86%.

PCR fingerprinting with prototype strains of *C neoformans* serotypes A, B, C, and D showed major diagnostic bands 15, 13, 17, and 18, respectively (figure 1). There were 86 patients with serotype A and one patient with serotype B (figure 2). Moreover, additional strains of *C neoformans* isolated from CSF, blood and urine from three patients and *C neoformans* cultured from two patients under treatment each week for 3 weeks, revealed the same serotype A.

In addition to tuberculosis and other parasitic infectious complications in this group of patients, a 35-year-old woman also had histoplasmosis and two men (30 and 33 years old) had penicillosis marneffei. Only four patients suffered from optic neuropathy; all were males and PCR fingerprinting revealed serotype A. There were no skin lesions in these patients.

The mean MICs of amphotericin B, flucytosine, fluconazole and itraconazole against the 87 isolates of *C neoformans* were 0.55 μg/ml (0.25–1, SD = 0.22), 9.5 μg/ml (2–20, SD = 4.91), 6.9 μg/ml (1–16, SD = 4.42) and 0.36 μg/ml (0.125–1.0, SD = 0.23), respectively.

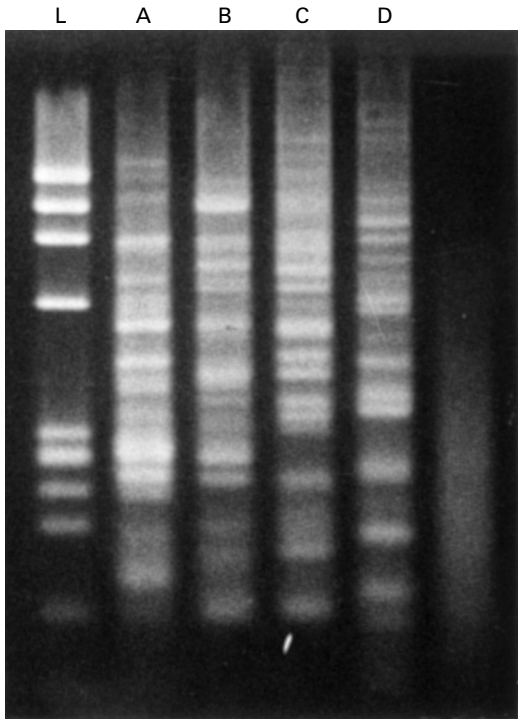

Figure 1 Electrophoretic separation of PCR fingerprints obtained after amplification of genomic DNA of four serotypes of *C neoformans* with the phage M13 core sequence. L = ϕxDNA/*Hae* III DNA marker

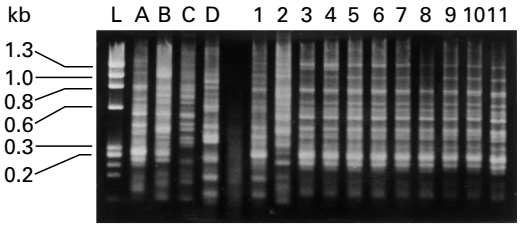

Figure 2 Electrophoretic separation of PCR fingerprints obtained after amplification of genomic DNA of four serotypes of *C neoformans* and *C neoformans* isolated from patients with the phage M13 core sequence. L = ϕxDNA/*Hae* III DNA marker

Discussion

From January 1996 to December 1997, 2643 129 patients attended the out-patient department and 119 951 patients were admitted to Siriraj Hospital; this provided a large population in which to conduct this study. From a retrospective study at Siriraj Hospital before the AIDS era, the ratio of incidence of cryptococcosis in female:male patients was 2:1, cryptococcosis being a common infection in systemic lupus erythematosus.[11–13] Nowadays it is regarded as a disease of sexually active people, primarily men with AIDS. The male-to-female ratio of the study patients was 4:1. This is in same range as the male-to-female ratio of HIV-infected patients in Thailand. In this study, the mean age of the patients was 32.1 years and mean CD4 lymphocyte count was 45 mm^3. HIV infection occurs predominantly in the lower economic social class. This, together with the tendency of people to present to the hospital late, are the main reasons why many of the fatalities took place after a mean of only 14 days hospitalisation, which was shorter than the mean duration of admission of survivors (26 days, $p < 0.001$). The majority of patients died of increased intracranial pressure with severe headache, convulsion and coma.

Some patients died of disseminated infection in lymph nodes, spleen and bone marrow.

The treatment of choice is amphotericin B (0.5–0.8 mg/kg/day). A combination of amphotericin B and flucytosine (150 mg/kg/day) is more effective than amphotericin B alone, but is associated with marrow suppression. Fluconazole (200–400 mg/day) can be used in patients whose signs and symptoms are not so severe and who can take medications orally. Secondary prophylaxis using amphotericin B (1 mg/kg) given intravenously every 1–2 weeks or fluconazole (100–200 mg/day) taken orally is recommended to preventing recurrent infection.

Although PCR has been used in the diagnosis of various diseases, especially infectious diseases, it has only recently been introduced to diagnose cryptococcosis. However, its efficiency is not superior to that of India ink preparation or latex agglutination, the most commonly used conventional methods. Moreover, conventional methods are easy to perform and less expensive than PCR, which needs specific primers and experienced operators.[14] The low titre of the latex agglutination test (1:16) in this study may be the result of early detection. In Thailand, where central nervous system fungal infection in HIV-infected patients is common, HIV-infected patients with neurological signs and symptoms are assessed promptly for cryptococcal infection.

In Papua New Guinea, in contrast to other countries, cryptococcal meningitis occurs predominantly in immunocompetent, previously healthy adults. *C neoformans* var *gattii* was implicated in 95% of cases and ocular complications were common.[15] A study in Italy revealed that the incidence of serotype D was 71% and skin lesions were observed only in serotype D infections.[16]

As in our previous investigation[17] and in that of Poonwan *et al*[18] on serotypes of *C neoformans* in Thailand, serotype A (*C neoformans* var *neoformans*) predominated in this study. This might explain why we had fewer problems with visual loss and skin lesions.

The susceptibility of *C neoformans* to antifungal agents corresponded very well with the results of other studies and all isolates were sensitive to the antifungal agents tested.[16 19–21]

Summary points

- cryptococcal meningitis in AIDS patients is a very common condition
- HIV in Thailand is more common in heterosexuals
- conventional methods (India ink preparation, latex agglutination test and culture) are reliable tests and the latex agglutination test is useful for monitoring patients
- *Cryptococcus neoformans* serotype A predominates (99% of patients)
- *in vitro* susceptibility tests against the *C neoformans* isolates showed them to be sensitive to the antifungals used

We would like to thank the representatives of Pfizer International Corp, Janssen Pharmaceuticals, Hoffman-La-Roche, and Bristol-Myers Squibb in Thailand for supplying fluconazole, itraconazole, flucytosine and amphotericin B, respectively. Dr Ira F Salkin (Albany, New York) provided strains of *C neoformans*, Dr Shusee Visalyaputra analysed the data, and Dr Timothy Mastro, Dr Warren Brockelmann, and Dr Bob Cowan, reviewed the manuscript. This research work was supported by a grant from Mahidol University and partially supported by the Siriraj-Chalermprakiat Fund.

1 Rippon JW. *Medical mycology. The pathogenic fungi and the pathogenic actinomycetes*, 2nd edn. Philadelphia: WB Saunders, 1982; pp 542–58.
2 Satitnimankarn T, Na Nakorn S, Rigunti M. Cryptococcosis, report of first case in Thailand. *J Med Assoc Thai* 1960; **43**:78–80.
3 Prariyachatigul C, Chaiprasert A, Meevootisom V, Pattanakitsakul S. Assessment of a PCR technique for the detection and identification of *Cryptococcus neoformans*. *J Med Vet Mycol* 1996;**34**:251–8.
4 Kwon-Chung KJ, Bennett JE. Epidemiologic differences between the two varieties of *Cryptococcus neoformans*. *Am J Epidemiol* 1984;**120**:123–30.
5 Salkin IF, Hurd NJ. New medium for differentiation of *Cryptococcus neoformans* serotype pairs. *J Clin Microbiol* 1982;**15**:169–71.
6 Tanaka K, Kohno S, Miyazaki T. The eiken latex test for detection of a cryptococcal antigen in cryptococcosis. *Mycopathologia* 1994;**127**:131–4.
7 Meyer W, Mitchell TG, Freedman EZ, Vilgalys R. Hybridization probes for conventional DNA fingerprinting used as single primers in the polymerase chain reaction to distinguish strains of *Cryptococcus neoformans*. *J Clin Microbiol* 1993;**31**:2274–80.
8 Viviani MA, Wen H, Roverselli A, *et al*. Identification by polymerase chain reaction fingerprinting of *Cryptococcus neoformans* serotype AD. J Med Vet Mycol 1997; 35:355–60.
9 Bowman BH. A model PCR/probe system for the identification of fungal pathogens. In: Perrsing DH, Smith TF, Tenover FC, White TJ, eds. *Diagnostic molecular microbiology. Principles and applications*. Washington, DC: American Society for Microbiology, 1993; pp 423–30.
10 National Committee for Clinical Laboratory Standards. *Reference method for broth dilution antifungal susceptibility testing for yeasts: proposed standard*. Document M27-P. Villanova, PA: National Committee for Clinical Laboratory Standards, 1992.
11 Leelarassami A, Danchaivichit S, Leelarassami I. Cryptococcosis : report of 19 cases from Siriraj Hospital. *Bull Inf Dis Gr Thai* 1982;**5**:52–62.
12 Imwidthaya P, Dithaprasop P. Egtasaeng C. Clinical and environmental isolates of *Cryptococcus neoformans* in Bangkok (Thailand). *Mycopathologia* 1989;**108**:65–7.
13 Imwidthaya P, Egtasaeng C. Latex agglutination test for diagnosing cryptococcosis. *J Med Assoc Thai* 1991;**74**:454–8.
14 Wu J, Liao W, Chai J. Isolation of specific DNA probes for detection of *Cryptococcus neoformans*. *Mycoses* 1997;**40**:385–9.
15 Seaton RA, Verma N, Naraqi S, Wembri JP, Warrell DA. Visual loss in immunocompetent patients with *Cryptococcus neoformans* var *gattii* meningitis. *Trans R Soc Trop Med Hyg* 1997;**91**:44–9.
16 Tortorano AM, Viviani MA, Rigoni AL, Cogliati M, Roverselli A, Pagano A. Prevalence of serotype D in *Cryptococcus neoformans* isolates from HIV positive and HIVnegative patients in Italy. *Mycoses* 1997;**40**:297–302.
17 Imwidthaya P, Sethakorn C, Chaiprasert A, Sutthent R, Poungvarin N. *Cryptococcus neoformans* in Siriraj Hospital 1994–1995. *Siriraj Hosp Gaz* 1997;**49**:1145–52.
18 Poonwan N, Mikami Y, Poosuwan S, *et al*. Serotyping of *Cryptococcus neoformans* strains isolated from clinical specimens in Thailand and their susceptibility to various antifungal agents. *Eur J Epidemiol* 1997;**13**:335–40.
19 Anaissie E, Shawar R, Paetznick V, *et al*. Two-site comparison of broth microdilution and semisolid agar dilution methods for susceptibility testing of *Cryptococus neoformans* in three media. *J Clin Microbiol* 1993;**31**:1370–2.
20 Barchiesi F, Colombo AL, McGough DA, Rinaldi MG. Comparative study of broth macrodilution and microdilution techniques for in vitro antifungal susceptibility testing of yeast by using the national committee for clinical laboratory standards' proposed standard. *J Clin Microbiol* 1994;**33**: 2494–500.
21 Ghannoum MA, Fu Y, Ibrahim AS, *et al*. *In vitro* determination of optimal antifungal combinations against *Cryptococcus neoformans* and *Candida albicans*. *Antimicrob Agents Chemother* 1995;**39**:2459–65.