CLONING OF cDNA CLONES ENCODING STRONGLY IMMUNOGENIC PROTEIN ANTIGENS FROM THE YEAST PHASE OF PENICILLIUM MARNEFFEI

BY

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ABSTRACT

Disseminated *Penicillium marneffei* infections have become increasingly prevalent, especially among human immunodeficiency virus-infected patients. We published the presence of IgG antibodies recognition of the yeast phase antigens of 200, 88, 54 and 50 kDa in *P. marneffei* infected AIDS patients. In order to provide recombinant antigens for further characterization, we tried to construct cDNA library encoding these antigens. Total RNA was isolated from the yeast form *P. marneffei* and the mRNA was purified by various methods. In this study, the yield of mRNA isolation was about 1.0% of a total RNA population. The SuperScript Lambda system (BRL) was used in constructing a directional cDNA library from an mRNA population. In step of *in vitro* packaging of ligated cDNA, the *in vitro* packaging efficiency of the control DNA was rather low ($<10^7$ PFU/μg of control DNA), and no plaque formation was seen in the test reaction. However, we plan to construct the cDNA library of *P. marneffei* by using paramagnetic beads and PCR.

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INTRODUCTION

Disseminated mycotic infections caused by the dimorphic fungus *Penicillium marneffei* have become increasingly prevalent, especially among AIDS patients (1-7). In nature, *P. marneffei* has been isolated from the internal organs of four species of rodents, *Rhizomys sinensis, Rhizomys pruinosus, Rhizomys sumatrensis,* and *Cannomys badius* (8-10). The fungus has been found in faeces of bamboo rats and soil samples collected from bamboo rat burrows (9,11). Two genetic types of *P. marneffei* have been identified by RFLP patterns of whole cell DNA (12). Type I predominates among infected patients (72.7%) and *Rhizomys sumatrensis,* whereas type II has been found in a soil sample, *Cannomys badius,* and in a minority of patients (27.3%).

The severity of *P. marneffei* infection in AIDS patients correlates with the immunological status of the individual. Most patients who do not receive appropriate antifungal treatment will die (6). The definite diagnosis of human penicilliosis is made by culture. The organism is often cultured from blood, skin, bone marrow, sputum, lymph nodes, cerebrospinal fluid and pleural fluid from AIDS patients infected with *P. marneffei.* The method requires 7 - 14 days and is difficult in patients who are concomittantly infected with other rapidly growing fungi or bacteria.

Currently, the identification of *P. marneffei* is based on the characteristic colony morphology, microscopic morphology and thermal dimorphism (13). However, these methods require approximately 2 - 4 weeks to accomplish complete identification. An exoantigen test (14,15) can also be used to confirm the identification of the fungus in culture. Newly developed immunodiffusion and latex agglutination tests are capable of detecting *P. marneffei* antigens in both serum and urine (16). In addition, a specific indirect fluorescent-antibody reagent was developed for the rapid identification of *P. marneffei* in histologic sections (17). *P. marneffei* infection can potentially be diagnosed by the PCR(18), the detection of IgG anibodies in the sera of patients using the indirect immunofluorescent antibody test (19) and the immunoblot assay (20, 22). Five yeast phase proteins of *P. marneffei* (approximately 88, 61, 54, 50 and 38-kDa) were found to react with IgG in sera of infected patients (20-22). These antigens may be useful as specific antigens in diagnostic serologic assay and further studies of the immunologic features of penicilliosis marneffei.

In order to provide recombinant antigens and a starting point of detailed structural analysis of immunogenic secreted proteins of *P. marneffei*, the cDNA encoding interesting protein antigens of *P. marneffei* yeast phase will be constructed.

MATERIALS AND METHODS

Microorganisms and growth conditions. *Penicillium marneffei* 496H isolated from a patient was used in this study. The fungus was cultured on brain-heart infusion (BHI) agar slant and incubated at 37^{0}C for 5 days to give a yeast form. This culture was further used as inoculum for the BHI broth culture. *Escherichia coli* Y1090 (ZL), from Gibco/BRL (Gaithersburg, MD, USA) was used for sereening the cDNA library. *E. coli* from the BACTi Disk was resuspended in 200 µl of sterile S.O.C. medium (Gibco/BRL) and incubated for 10 min at 37^{0}C. The bacterial solution was streaked on LB [1% (wt/vol) tryptone, 0.5% (wt/vol) yeast extract, 1% (wt/vol) sodium chloride and 1.5% (wt/vol) agar pH 7.0] plate and incubated overnight at 37^{0}C. A single colony of *E. coli* Y1090(ZL) was inoculated into 10-ml of L-broth containing 0.2% (w/v) maltose and 10 mM $MgSO_4$ on the day before ligated vector-cDNA was to be packaged in vitro. The culture was incubated at 37^{0}C overnight. If the cells were not used the same day they could be stored at 4^{0}C for a maximum of 3 days.

Preparation of total RNA of *P. marneffei*. Three-day *P. marneffei* yeast-form culture was centrifuged at 3,000 x g for 15 min, washed twice with phosphate-buffered saline, and washed once with 0.6 M $MgSO_4$. The yeast cells were suspended in filter-sterilized osmotic medium [1.2M $MgSO_4$, 10mM sodium phosphate (pH5.8); 5 ml/g of yeast cells], and the suspension was placed on ice. A filter-sterilized solution of Novozym234 (20 mg/ml in osmotic medium; 2 ml/g of yeast cells) was added, and the cells were incubated on ice for 5 min. Next, a filter-strilized solution of bovine serum albumin (12mg/ml in osmotic medium; 0.5 ml/g of yeast cells) was added. The suspension was then incubated at 37^{0}C for 2 h. The spheroplasts were pelleted by centrifugation, washed twice with ST buffer [0.6 M sorbitol, 100 mM Tris-HCl (pH 7.0)]. Approximately 1.6-2.0 g wet weight of yeast spheroplasts were obtained from 500 ml culture (about 10g wet weight of yeast cells).

Tolal RNA was extracted from yeast spheroplasts by using guanidine isothiocyanate in the presence of 2-mercapto ethanol which were included in the mRNA isolation system (BRL, Gaithersburg, MD, USA) or by RNAgents® total RNA isolation system (Promega, Madison, WI, USA) or by TRIzol ™ reagent (BRL) or by RNeasy minipreps (Qiagen GmbH, Hilden, Germany) following the manufacturers' instructions.

Preparation of mRNA. mRNA of *P. marneffei* was isolated by the mRNA isolation system (BRL) or by PolyATract mRNA isolation system (Promega) following the manufacturers' instructions. The principle of mRNA isolation based on the presence of poly (A) tails at their 3' termini. The mRNA could be isolated from a total RNA population by affinity chromatography using column containing oligo (dT)

cellulose in the presence of 0.3 to 0.5 M NaCl (BRL). The mRNA was eluted by destabilizing the hybrids of oligo dT and poly (A). This was done by removing the NaCl. This method could yield >90% mRNA. Another method of mRNA isolation system (Promega) used a biotinylated oligo(dT) primer to hybridize to the 3' poly (A) region. The hybrids were captured and washed at high stringency using streptavidin coupled to paramagnetic particles and a magnetic separation stand. The mRNA was eluted by the simple addition of ribonuclease free deionized water. Figure 1 summarizes all steps in the procedure of total RNA and mRNA isolation by the system provided by the BRL. Figure 2 shows the diagram of the PolyATract® mRNA isolation procedure provided by the Promega.

Construction of cDNA library. The SuperScript Lambda system (BRL) was used in constructing a directional cDNA library from an mRNA population of *P. mameffei*. The procedure was summarized in Figure 3 and the flow diagram in Figure 4. First strand cDNA was synthesized using SuperScript RT [(Maloney merine leukemia virus reverse transcriptase (M-MLV RT)] and oligo (dT) (*Not* I primer adapter) as a primer. The second strand cDNA synthesis was catalyzed by *E. coli* DNA polymerase I in combination with *E. coli* RNase H and *E. coli* DNA ligase. The blunt end of cDNA was done by adding T4 DNA polymerase. Next the *Sal* I adapters were added at both ends of the cDNA prior to digestion with *Not* I to get *Not* I *Sal* I cDNA. The optimal sizes of cDNA were fractionated by column chromatography. The cDNA were then ligated to the vector lambda Ziplox, *Not* I-*Sal* I arms. The ligated cDNA were introduced into the lambda bacteriophage *E. coli* Y1090 (ZL) using the Amersham (Buckinghamshire, UK) lambda Packaging system. The size of the library could be estimated by plaque assay.

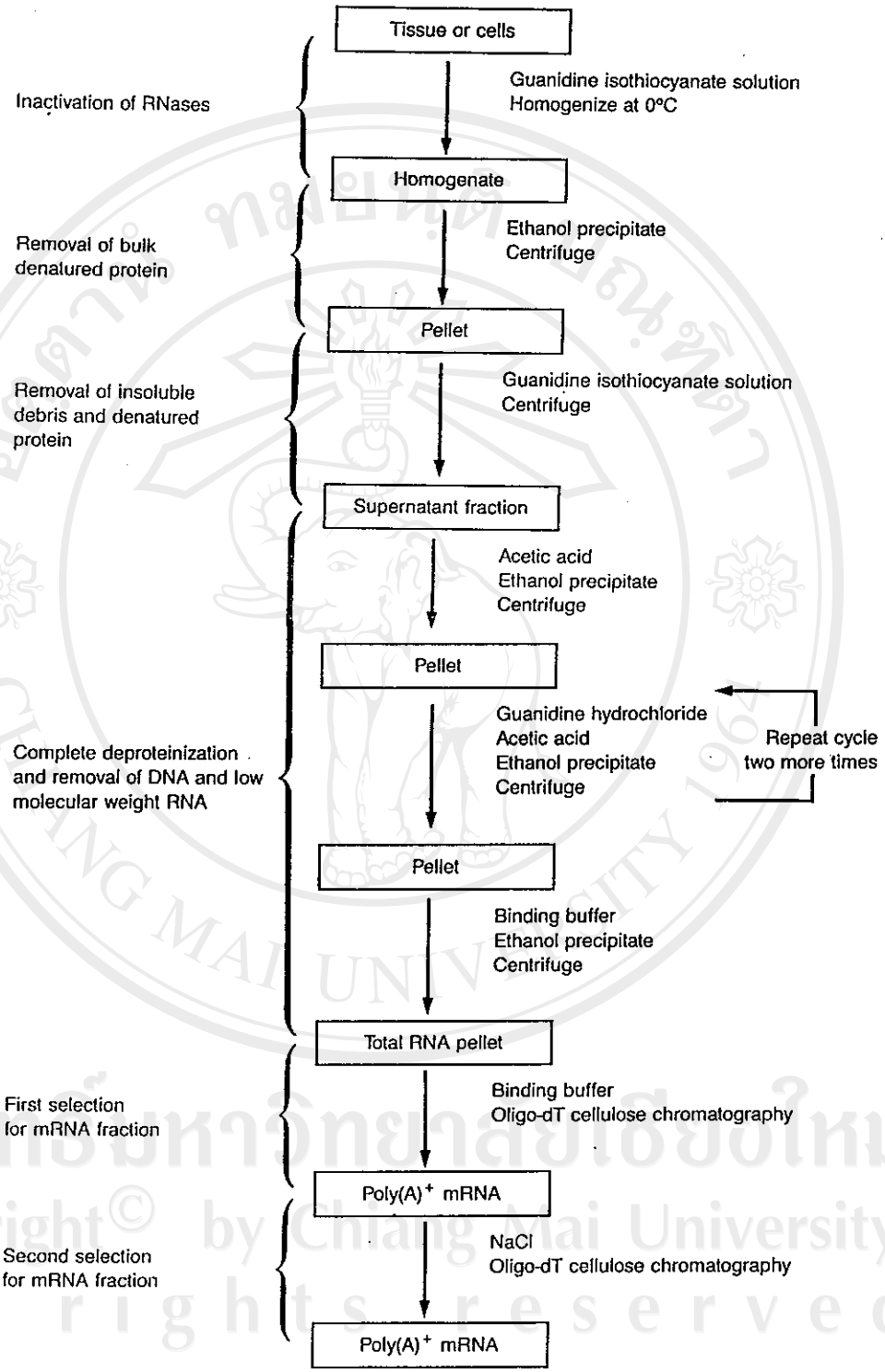

Figure 1. Isolation of total RNA and mRNA by the mRNA isolation system (BRL).

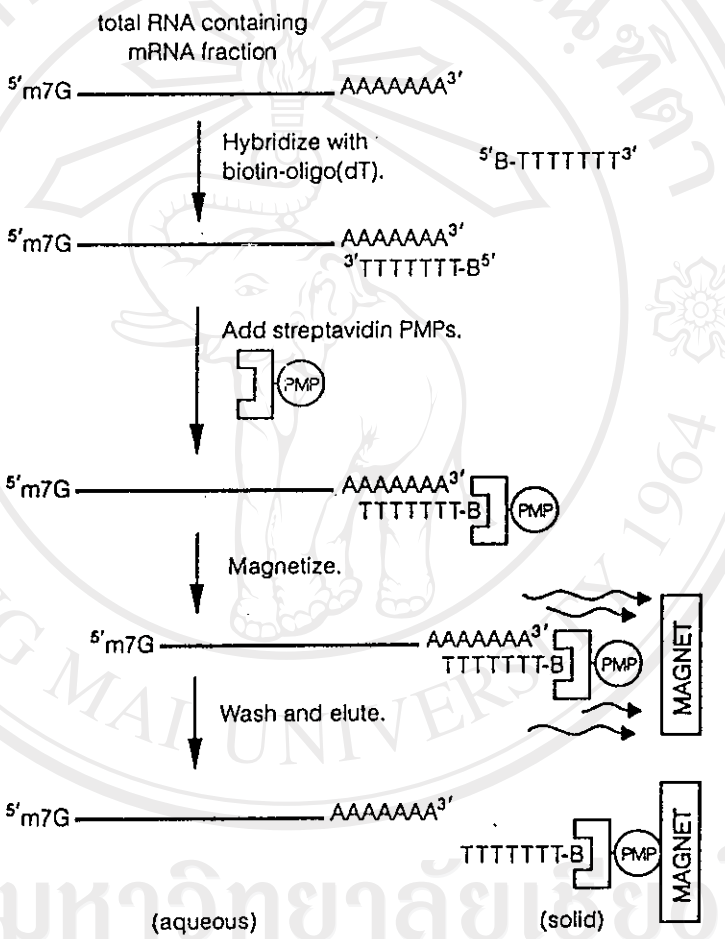

Figure 2. The diagram of mRNA isolation procedure by the Poly ATract mRNA isolation system (Promega).

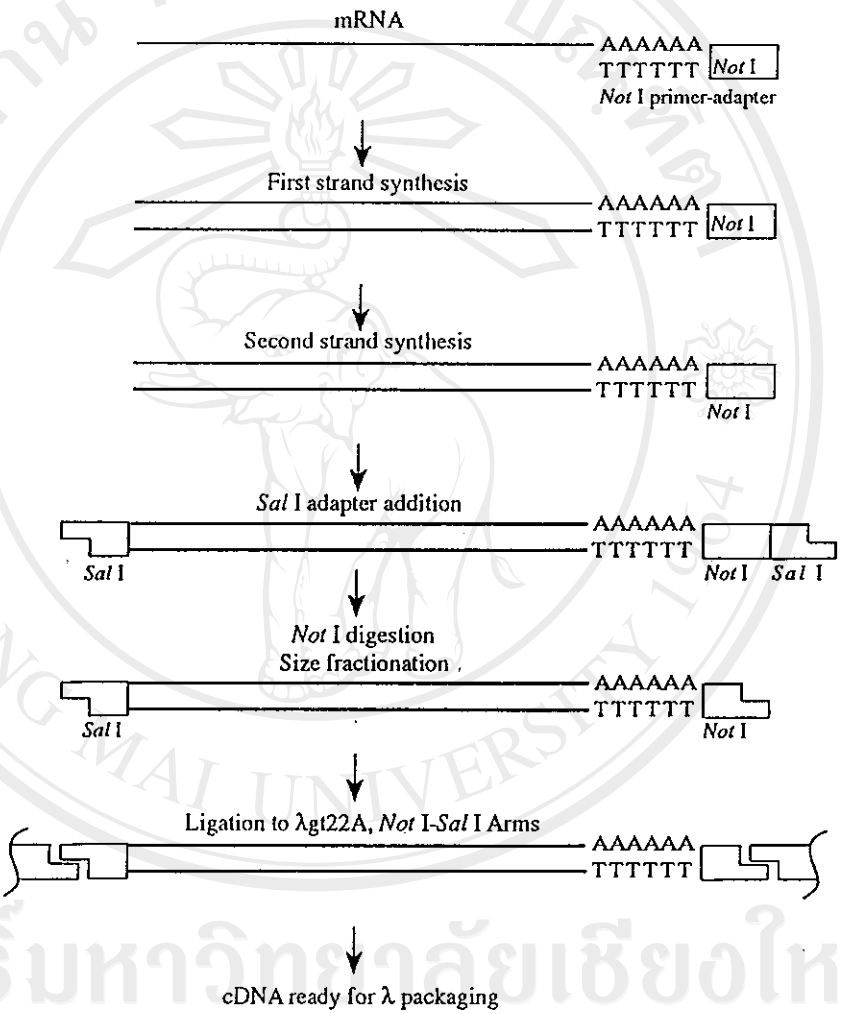

Figure 3. Flow diagram of the directional cDNA library construction by the SuperScript Lambda system (BRL).

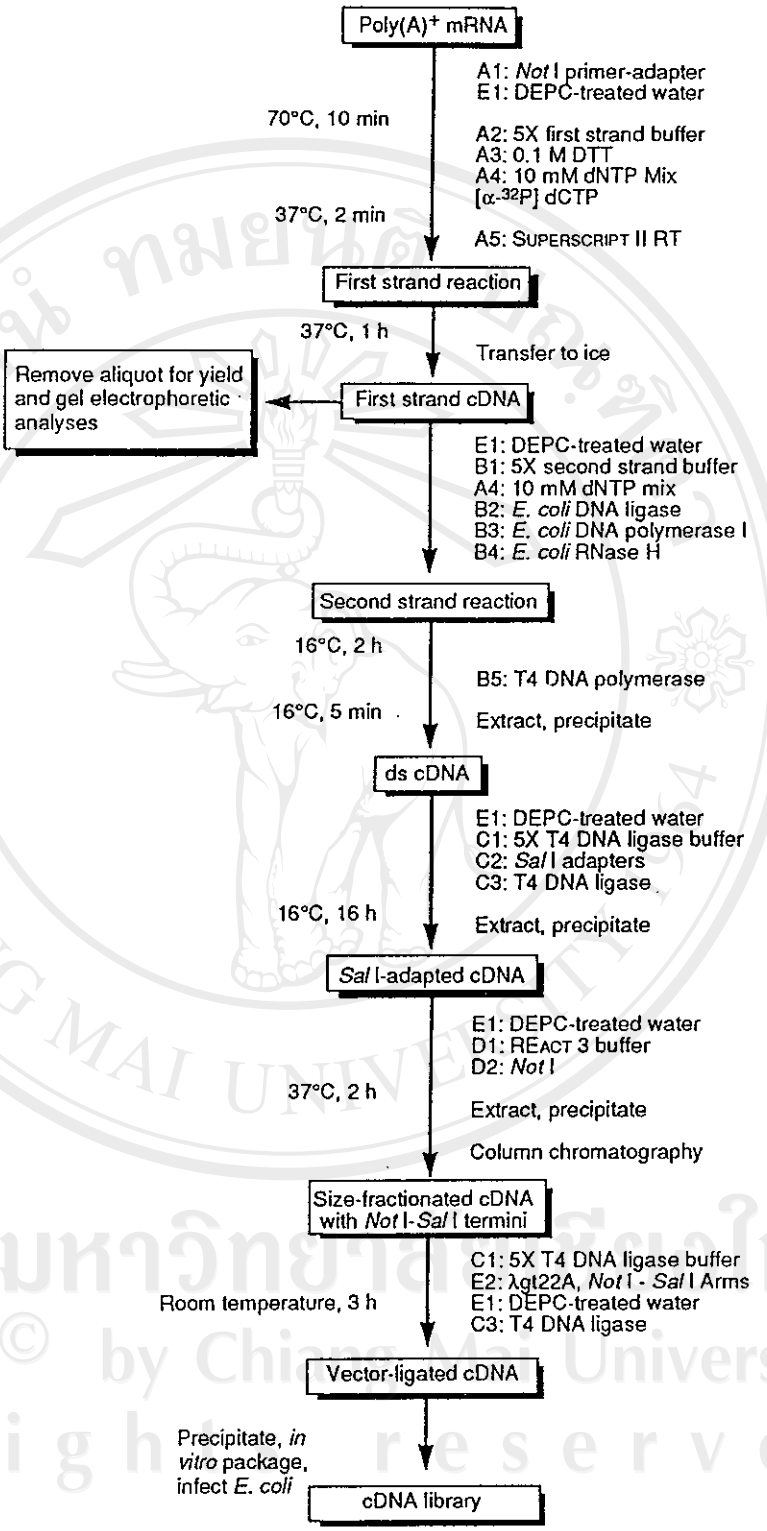

Figure 4. Detailed protocol flow diagram of cDNA library construction.

RESULTS AND DISCUSSIONS

The total RNA isolation systems used in this study yielded high amounts of intact RNA from the *Penicillium marneffei* yeast form spheroplasts. The yield was determined by measuring the optical density at 260 nm, where 1 A 260 unit = 40 μg of RNA/ml. The results were approximately 1.5 mg and 3.4 mg of total RNA from 500 ml culture using the mRNA isolation system (BRL) and RNAgents total RNA isolation system (Promega), respectively. Total RNA was easily isolated using the RNeasy minipreps (Qiagen). The yield was about 1.0 mg of total RNA from 1.0 g of fungal spheroplasts (or from about 500 ml culture broth). Figure 5 demonstrates formaldehyde agarose gel of total RNA purified with the RNeasy mini kit and by TRIzol™ reagent.The RNAs isolated by the RNeasy mini kit were more purified than the RNAs isolated by TRIzol™ reagent.The 28S and 18S rRNAs are seen as sharp bands (Fig. 5) with a diffuse background mRNA spread throughout the entire lane of the gel.

The mRNA purified by the affinity column [oligo (dT) cellulose from BRL] is demonstrated in Figure 6 by denaturing agarose gel staining with ethidium bromide. Figure 7 shows the mRNA purified by the PolyATract of Promega. Typically, approximately 0.5% to 2.0% of a total RNA population is mRNA. In this study, the yield of mRNA was about 1.0%

In step of cDNA ligation to the vector, lambda Ziplox (BRL) was used instead of the lambda gt 22A. The lambda Ziplox *Not* I-*Sal* I arms were provided for directional cloning. The control DNA included in this system was used parallel in the ligation step. After the *in vitro* packaging of ligated cDNA, the packaging reaction was introduced into *E. coli* Y1090 (ZL). The packaging cDNA-containing phage and the packaged control DNA-containing phage were tested by dilution plagues assay on the LB plates containing 10mM $MgCl_2$. The number of plagues of the control DNA (0.1 μg) were calculated to be approximately 10^6 PFU from the entire packaging reaction whereas no plaque was detected in the test reaction.

The *in vitro* packaging efficiency of the control DNA was rather low ($<10^7$ PFU/ μg of control DNA). This control DNA was used from the ligation step. It meaned that the ligation reaction may not be proceeding properly. Another cause of low packaging efficiency of the control DNA may involve with the extracts included in the packaging reaction which may lost some activity during performing the test. For the negative plaque formation of the test reaction from the synthesized cDNA, the poor yields may occur in the first strand reaction, or second strand reaction. An insufficient or very low amount of cDNA used in the ligation reaction may cause a very low packaging efficiency.

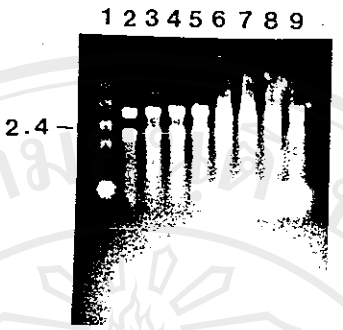

Figure 5. Formaldehyde agarose gel of total RNA purified with the RNeasy mini kit (lane 2-5), and by TRIzol reagent (lane 6-9). Lane 1, RNA ladder.

Figure 6. Formaldehyde agarose gel of mRNA purified by oligo (dT) cellulose column from BRL (lane 2). Lane 1, marker sizes of *Hind* III-digested lambda DNA.

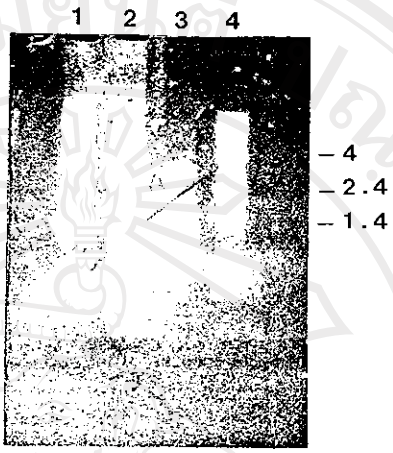

Figure 7. Formaldehyde agarose gel of mRNA purfied by PolyATract of Promega (lane 3). Lane 2, tolal RNA isolated by RNAgents of Promega. Lane 1 and lane 4, RNA ladder.

A lambda ZAP cDNA expression library of *P. marneffei* could be constructed and screened with human serum obtained from a penicilliosis patient (Cao et al. 1998). The approach proved to be difficult because the human serum produced a high background in the filter screening assay. Screening with an animal hyperimmune serum generated by immunizing guinea pigs with killed *P. marneffei* yeast cells gave better results. The MP1 gene which encodes an abundant antigenic cell wall mannoprotein from the *P. marneffei* was cloned. However, we plan to construct the cDNA library of *P. marneffei* by using paramagnetic beads and PCR (Lambert and Williamson, 1997.) The screening procedure may use the patients' sera or rabbit hyperimmune serum.

REFERENCES

1. Chariyalertsak S, Sirisanthana T, Supparatpinyo K, Nelson KE. Seasonal variation of disseminated *Penicillium marneffei* infections in Northern Thailand: A clue to the reservoir? *J Infect Dis* 1996; **173:** 1490-3.
2. Chiewchanvit S, Mahanupab P, Hirunsri P, Vanittanakom N. Cutaneous manifestations of disseminated *Penicillium marneffei* mycosis in five HIV-infected patients. *Mycoses* 1991; **34:** 245-9.
3. Hilmarsdottir I, Meynard JL, Rogeaux O, Guermonprez G, Datry A, Katlama C, Bruecker G, Coutellier A, Danis M, Gentilini M. Disseminated *Penicillium marneffei* infection associated with human immuno-deficiency virus: a report of two cases and a review of 35 published cases. *J Acquired Immune Defic Syndr* 1993; **6:** 466-71.
4. Sirisanthana V, Sirisanthana T. *Penicillium marneffei* infection in children infected with human immunodeficiency virus. *Pediatr Infect Dis J* 1993; **12:** 12021-5.
5. Supparatpinyo K, Chiewchanvit S, Hirunsri P, Uthammachai C, Nelson KE, Sirisanthana T. *Penicillium marneffei* infection in patients infected with human immunodeficiency virus. *Clin Infect Dis* 1992; **14:** 871-4.
6. Supparatpinyo K, Khamwan C, Baosoung V, Nelson KE, Sirisanthana T. Disseminated *Penicillium marneffei* infection in Southeast Asia. *Lancet* 1994; **344:** 110-3.
7. Viviani MA, Tortorano AM, Rizzardini G, Quirino T, Kaufman L, Padhye AA, Ajello L. Treatment and serological studies of an Italian case of penicilliosis marneffei contracted in Thailand by a drug addict infected with the human immunodeficiency virus. *Eur J Epidemiol* 1993; **9:** 79-85.
8. Ajello L, Padhye AA, Sukroongreung S, Nilakul CH, Tantimavanic S. Occurrence of *Penicillium marneffei* infections among wild bamboo rats in Thailand. *Mycopathology* 1995; **131:** 1-8.
9. Chariyalertsak S, Vanittanakom P, Nelson KE, Sirisanthana T, Vanittanakom N. *Rhizomys sumatrensis* and *Cannomys badius*, new natural animal hosts of *Penicillium marneffei*. *J Med Vet Mycol* 1996; **34:** 105-10.

10. Deng Z, Yun M, Ajello L. Human penicilliosis marneffei and its relation to the bamboo rat (*Rhizomys pruinosus*). *J Med Vet Mycol* 1986; **24:** 383-9.

11. Deng ZL, Ribas JL, Gibson DW, Connor DH. Infections caused by *Penicillium marneffei* in China and Southeast Asia. Review of eighteen cases and report of four more Chinese cases. *Rev Infect Dis* 1988; **10:** 640-52.

12. Vanittanakom N, Cooper CR, Chariyalertsak S, Youngchim S, Nelson KE, Sirisanthana T. Restriction endonuclease analysis of *Penicillium marneffei*. *J Clin Microbiol* 1996; **34:** 1834-6.

13. Segretain G. *Penicillium marneffei* n. sp., agent d'une mycose du systeme reticuloendothelial. *Mycopathol Mycol Appl* 1959; **11:** 327-53.

14. Sekhon AS, Li JSK, Garg AK. Penicilliosis marneffei: serological and exoantigen studies. *Mycopathology* 1982; **77:** 51-7.

15. Sekhon AS, Garg AK, Padhye AA. Antigenic relationship of *Penicillium marneffei* to *P. primulinum*. *J Med Vet Mycol* 1989; **27:** 105-12.

16. Kaufman L, Standard PG, Jalbert M, Kantipong P, Limpakarnjanarat K, Mastro TD. Diagnostic antigenemia tests for penicilliosis marneffei. *J Clin Microbiol* 1996; **34:** 2503-5.

17. Kaufman L, Standard PG, Anderson SA, Jalbert M, Swisher BL. Development of specific fluorescent-antibody test for tissue form of *Penicillium marneffei*. *J Clin Microbiol* 1995; **33:** 2136-8.

18. Vanittanakom N, Merz WG, Sittisombut N, Khamwan C, Nelson KE, Sirisanthana T, Specific identification of *Penicillium marneffei* by a polymerase chain reaction/hybridization technique. *Med Mycol* 1998, in press.

19. Yuen K, Wong SS, Tsang DN, Chau P. Serodiagnosis of *Penicillium marneffei* infection. *Lancet* 1994; **344:** 444-5.

20. Vanittanakom N, Mekaprateep M, Sittisombut N, Supparatpinyo K, Khanjanasthiti P, Nelson KE, Sirisanthana T. Western immunoblot analysis of protein antigens of *Penicillium marneffei*. *J Med Vet Mycol* 1997; **35:** 123-31.

21. Chongtrakool P, Chaiyaroj SC, Vithayasai V, Trawatcharegon, Teanpaisan R, Kalnawakul S, Sirisinha S. Immunoreactivity of a 38-kilodalton *Penicillium marneffei* antigen with human immunodeficiency virus-positive sera. *J Clin Microbiol* 1997; **35:** 2220-3.

22. Jeavons L, Hamilton AJ, Vanittanakom N, Ungpakorn R, Evans EGV, Sirisanthana T, Hay RJ. Identification and purification of specific *Penicillium marneffei* antigens and their recognition by human immune sera. *J. Clin Microbiol* 1998; **36.** 949-54.

23. Cao L, Chan CM, Lee C, Wong SS, Yuen KY. MP1 encodes an abundant and highly antigenic fungus *Penicillium marneffei*. *Infect Immun* 1998, **66** : 966-73.

24. Lambert KN, Williamson VM. cDNA library construction from small amounts of RNA using paramagnetic beads and PCR. In : IG Cowell, CA Austin (eds) Methods in Molecular Biology, Vol 69 : cDNA library protcols, 1997, pp 1-12.