V. DISCUSSION

Diagnosis of disseminated penicilliosis due to *P. marneffei* by using conventional culture usually takes 7-14 days. The effective treatments of *P. marneffei* infection are often late. Rapid diagnosis is important, because disseminated *P. marneffei* infection has a high mortality, and effective antifungal treatments are available (Supparatpinyo et al, 1992). Identification of *P. marneffei* by means of an immunohistochemical approach (Estrada et al, 1992) and exoantigen test (Sekhon et al, 1982) were reported; however, these tests were also time-consuming and were not generally available. A number of serological diagnosis tests have been developed for the detection of antibody to *P. marneffei*. These include an immunodiffusion (Sekhon et al, 1982; Imwidthaya et al, 1997), indirect immunofluorescent antibody technique (Yuen et al, 1994) and immunofluorescent test (Kaufman et al, 1995). The usefulness of available tests for antibody detection has been limited by their low sensitivity and specificity by using the crude antigens. Cross reactivity between fungal pathogens can lead to incorrect diagnosis. However, specific antigens of *P. marneffei* were identified by Western blot assays (Chongtrakool et al, 1997; Vanittanakom et al, 1997A; Jeavons et al, 1998). The Western blot analysis revealed that the specific antigens of *P. marneffei* were recognized with penicilliosis sera. Recently, the recombinant antigen, Mp1p which was expressed from the *MP1* gene of *P. marneffei*, was used to detect antibody in sera from penicilliosis patients by ELISA method (Cao et al, 1998B; Cao et al, 1999). However, rabbit anti-Mp1p antibody could not react specifically with the protein antigens of *P. marneffei* Thai isolates (Unpublished data). Therefore, this test may not be useful for detection *P. marneffei* infection in Thailand.

In this study, two potential antigenic proteins of *P. marneffei* from cDNA library, *P6* and *P23* were selected. These genes encoding antigenic proteins in the pathogenic fungus *P. marneffei* which were screened by using pooled sera from patients infected with *P. marneffei* (Pongpom, 2004). BLAST analysis was performed to search for homologs suggesting the potential biological functions of *P6* and

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P23. The BLAST results suggest that *P6* is a novel gene without homolog in any published gene database. The *P23* encoding 187 amino acid residues contains features similar to several 30-kDa heat shock proteins. The distribution of polar and apolar residues along a protein sequence is displayed by hydrophobicity plots. The hydrophobicity of the amino acid determines where the amino acid will be located in the final structure of the protein. From the protein sequence, it was predicted that *P6* has highly hydrophilic regions near the N-terminus (**Figure 9A**) and *P23* has highly hydrophilic domains in a whole sequence (**Figure 10A**). Both proteins contain prediction of antigenic domains (**Figures 9B and 10B**). Hydrophilic regions that are likely exposed on the surface may possibly be antigenic. Prediction of post-translational modification showed that *P6* contains N-glycosylated site at asparagine position 163 and a serine- and threonine-rich region for O-glycosylation. *P23* contains a serine- and threonine-rich region for O-glycosylation.

In this study, the vector pGEX-4T-1 was chosen. The pGEX-4T-1 is one of vector in series of pGEX vectors which are among the most popular expression vectors that give a fusion product (Smith et al, 1988). The *tac* promoter is placed upstream of a lengthy open reading frame coding for most of the GST gene. The cloning sites lie immediately downstream of this open reading frame, arranged in such a way that insertion of an appropriate DNA fragment leads to synthesis of a fusion protein (**Figure 14**). Insert DNA should be less than 2 kb long. In this study, the insert DNA was 0.56 kb long. Cao and collaborators (1998) were successful in using the vector in pGEX series, pGEX30. The *MP1* gene of *P. marneffei* was amplified and cloned into the expression vector, pGEX30. The GST-Mp1p fusion protein was expressed and purified with the GST gene fusion system (Pharmacia). The results from Cao and his colleagues revealed that the vector in pGEX series could be applied in the expression study of fungal antigens.

Construction of the recombinant plasmid, the PCR products amplified from *P6* and *P23* cDNA and the pGEX-4T-1 were digested with *Xho* I and *Bam* HI to generate two cohesive ends as described in the Materials and Methods. Then, the digested PCR products and digested pGEX-4T-1 were ligated together with molar ratio of insert and vector, 5:1 (GST Fusion Protein Handbook, Amersham Biosciences, Sweden). The recombinant plasmids were transformed into the competent *E. coli* host. The

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recombinant clones were checked for the presence of inserted DNA by *Xho* I and *Bam* HI digestion. The recombinant plasmid of each product showed the expected restriction pattern; two bands of approximately 0.56 and 4.96 kb (**Figures 20 and 21**). The results confirmed that the recombinant clones contained the desired DNA fragments. The 561 bp of the insert DNA sequences of *P6* and *P23* were confirmed by DNA sequencing. The nucleotide sequence analysis revealed correct sequence of the interesting DNA fragment of *P6* or *P23* ligated directionally in-framed with the GST sequence in the pGEX-4T-1.

Further, the recombinant plasmids of pGEX-*P6* and pGEX-*P23* or the plasmid vector, pGEX-4T-1 were transformed into *E. coli* BL21 and induced with IPTG to express the GST proteins or GST fusion protein in a small scale culture. The following conditions were optimized to express high level of soluble protein: the growth temperature (25 °C) to improve solubility, IPTG concentration of < 100 μM to alter induction level, a higher cell density to induce for a short period of time and increase aeration (high oxygen transport can help prevent the formation of inclusion bodies). The bacterial pellets of an induced culture were resuspended in PBS buffer pH 7.2 containing protease inhibitors. The suspension was lysed by sonication and centrifuged to collect the supernatant containing the soluble form of the recombinant protein for purification.

Most protein synthesis vectors are designed in such a way that the protein is expressed as a fusion with a second protein, the latter offering some means for convenient purification from the protein extract usually by an affinity-binding procedure. Purification of the fusion protein makes use of the high affinity of GST for its natural substrate, glutathione. In this study the fusion proteins were purified by affinity chromatography using the immobilized glutathione. Then, the purified fusion proteins were analysed by SDS-PAGE and Western blot assays. In this experiment, it was found that the fusion proteins in the eluate fraction no.2 (E2) had a protein concentration in the highest level (**Figures 25 and 26, lanes E2**). Yield of the purified GST-P6p was approximately 1.89 mg, while yield of the purified GST-P23p was approximately 1.44 mg from the culture volume 200 ml (**Table 3**). The yield of GST-fusion proteins were higher than those protein concentration recommended in the GST Fusion Protein Handbook (Amersham Biosciences, Sweden). The

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recommendation yield of the purified GST-fusion protein was 0.5 mg per 200 ml culture volume.

To evaluate the properties of recombinant proteins, two methods are utilized for detection of GST fusion proteins, SDS-PAGE analysis and Western blot analysis using anti-GST antibody. In this study, parental pGEX-4T–1 vector produced a 27-kDa of GST protein. SDS-PAGE analysis for the purified GST-P6p showed three protein bands on the gel at molecular wieghts of 58, 34 and 32 kDa compared to the expected molecular weight of 47.2 kDa (**Figure 25 and Table 2**). The purified GST-P23p showed a single band on the gel at molecular weight of 53 kD, higher than the expected molecular weight of 47.7 kDa (**Figure 26 and Table 2**). Therefore, the MW of the GST-fusion proteins are different from the predicted MW. The protein bands of 34 and 32 kDa which were observed from the purified GST-P6p suggested that there may be impurity of the *E. coli* carrying the *P6* recombinant plasmid. There may be a mixture of recombinant plasmids, one of them might contain pGEX-*P6* with all *P6* coding region, while the other might contain pGEX-P6 with mutation probably generated during PCR amplification. To test this possibility, bacteria containing *P6* recombinant plasmid should be re-isolated on the LB containing 100 μg/ml of ampicillin. Isolated colonies will be randomly picked, cultured, purified and checked for protein expression.

To confirm whether proteins expressed from recombinant plasmids are GST-fusion proteins, GST-P6p, GST-P23p, GST protein and *E.coli* BL21 lysates were separated and transferred to nitrocellulose membrane. The membrane was then incubated with HRP-anti GST, and detected with chromogenic substrate. The detection of GST fusion proteins showed positive band at a molecular weight of 27 kDa for purified GST protein, three positive bands at molecular weights of 58, 34, and 32 kDa for purified GST-P6p and single positive band at molecular weight of 53 kDa for purified GST-P23p (**Figure 28**). The crude *E. coli* BL21 proteins showed negative reaction. The positive reactivities of the recombinant proteins with specific antibody, HRP-anti GST confirmed that the recombinant proteins are the GST-fusion proteins.

Immunoreactivities of the recombinant proteins were tested using sera from immunized rabbits and from patients with penicilliosis. The Western blot analysis of the recombinant proteins, GST-P6p showed positive band of 58-kDa with the serum

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from one immunized rabbit (rabbit no.1) (**Figure 30A, lane 2**). This rabbit was immunized with cytoplasmic antigens of *P. marneffei.* The reactivity was negative with the rabbit antiserum against concentrated secreted antigens of *P. marneffei* (rabbit Y1) or another rabbit antiserum against concentrated cytoplasmic antigens of *P. marneffei* (rabbit no.2) (**Figure 30A, lanes 1 and 3**). The GST-P6p showed negative reaction with rabbit antiserum against concentrated cytoplasmic antigens of *C. neoformans* or *H. capsulatum* (at serum dilution 1:100) (**Figure 30A, lanes 4 and 5**). The data show that the animal model could produce specific antibody that recognized GST-P6p protein, while the recombinant proteins of GST-P23p and GST showed negative reaction (**Figures 30B and C**). The study of immunoreactivities with rabbits antisera showed very limited cross-reactivity with antibodies to different fungal pathogens, *C. neoformans* and *H. capsulatum.* However, the sensitivity and specificity of the antibody test with a pure recombinant protein require further investigations with larger number of sera from patients infected with *P. marneffei* and serum from patients infected with others fungal pathogen.

The Western blot analysis of the GST-P6p fusion protein showed positive reaction in 2 from 10 sera of the patients infected with *P. marneffei,* while sera of 10 normal laboratory personnels gave negative results at serum dilution 1:100 (**Figure 34A**). The analysis of anti-P6p antibodies in AIDS patients with penicilliosis marneffei revealed that only 20% of the patients have detectable levels of anti-P6p antibody. The Western blot analysis of the GST-P23p fusion protein showed positive reaction in other 2 sera from these 10 cases (**Figure 35**). The finding of low positive reaction suggested that perhaps several AIDS patients in this study did not produce detectable level of antibody to P6p or P23p. Although, the sensitivity of each GST-fusion protein determining by Western blot analysis is low, combining of these two fusion proteins would increase the positive reactivity to be 40% (4 of 10). The sensitivity of the GST-fusion proteins can be improved by increasing the concentration of fusion proteins and/or the dilution of serum samples. An antibody test could be more informative for patients with better humoral immunity. It could be useful when the fungal load is low (Cao et al.,1998). However, with lower immunity and increased fungal load, an antigenemia test would be more useful. Therefore, it should be noted that both antibody and antigen tests are needed for the serodiagnosis

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of penicilliosis to improve the sensitivity of the test. However, a large number of sera from *P. marneffei*-infected patients have to be tested and the specificity of both recombinant proteins requires extensive investigation.

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