Application of Immunoblot Assay for Rapid Diagnosis of Human Pythiosis

Jidapa Supabandhu PhD*, Pramote Vanittanakom Dr. rer.nat**,
Kamphol Laohapensang MD***, Nongnuch Vanittanakom Dr. rer.nat*

** Department of Microbiology, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand*
*** Department of Pathology, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand*
**** Department of Surgery, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand*

Objective: *Pythiosis, a life-threatening infectious disease, has been reported from Maharaj Nakorn Chiang Mai hospital since 2001. Delayed diagnosis, due to difficulty in obtaining an appropriate specimen and in timely identification, causes delayed treatment resulting in a high mortality rate. To address this problem, a previously developed immunoblot has been evaluated and objected as an effective diagnostic tool for human pythiosis in this hospital.*

Material and Method: *The immunoblot assay was evaluated using human sera with culturally proven fungal infection. Sera from humans with a variety of other fungal infections, pooled healthy human sera including Cryptococcus neoformans-, Penicillium marneffei-, and Histoplasma capsulatum-immunized rabbit antisera were used as controls. The assay was applied to evaluate twenty-six sera of suspected pythiosis patients. Moreover, in appropriate cases, a combination of immunoblot, culturing and polymerase chain reaction (PCR) were also performed in order to determine the accuracy of the immunoblot assay.*

Results: *The presented immunoblot assay was not reactive with any sera of the controls or those of the other fungal-infected patients used in the present study. Pythiosis could be differentiated from other fungal infections with similar symptoms in sixteen of twenty-six samples of suspected patients. The positive ones showed the proper reactive pattern as shown in P. insidiosum-immunized rabbit serum. The present study provided evidence that the 40 to 35-kDa antigens were reactive specifically with all sera from both treated and active pythiosis patients. Culturing and PCR results were consistent with the immunoblot finding.*

Conclusion: *The immunoblot assay developed in the present study is specific to P. insidiosum-infection and suitably applied as an effective tool for human pythiosis diagnosis.*

Keywords: *Diagnosis, Immunoblotting, Infection, Pythium*

J Med Assoc Thai 2009; 92 (8): 1063-71
Full text. e-Journal: *http://www.mat.or.th/journal*

Human pythiosis is a life-threatening disease caused by the mammalian pathogen *Pythium insidiosum*. Human pythiosis has been documented mainly in Thailand and occasionally in other tropical and subtropical countries such as Australia, Haiti, New Zealand, and the USA. The first five cases of human pythiosis were found in rural areas of northern Thailand[1]. More recently, however, pythiosis cases have been reported from every region of Thailand[2,3]. Clinical manifestations of the disease have been described in four forms, including cutaneous/subcutaneous[4], ocular[5,6], vascular, and disseminated forms[3,7]. Pythiosis has been reported both in patients with immunological abnormalities and in apparently healthy individuals. Patients with cutaneous/subcutaneous, disseminated and vascular forms are usually associated with hematological disorders, such as thalassemia, leukemia or pyroxysmal nocturnal hemoglobinuria[2,7,8]. In contrast, most ocular cases are usually found in healthy individuals[5,9].

Correspondence to: Vanittanakom N, Department of Microbiology, Faculty of Medicine, Chiang Mai University, Chiang Mai 50200, Thailand. Phone: 053-945-332, Fax: 053-217-144. E-mail: drnongnuch@gmail.com

Working in aquatic habitats are the most likely risk factors involved in human pythiosis. The natural occurrence of *P. insidiosum* has recently been determined[10]. Irrigation water may be the prevalent source of *P. insidiosum* colonization and infection, especially for individuals working in endemic agricultural areas[10]. In its natural aquatic habitat, *P. insidiosum* produces motile biflagellate zoospores that likely play an important role in the initiation of animal infection[11]. Damaged tissue and open wounds could attract zoospores to bind and attach to them using an adhesive material.

In human pythiosis, the disseminated and vascular forms are lethal, since they have a very high mortality rate. Although the ocular form is not life-threatening, eye recovery has not been entirely successful[13]. Therefore, rapid and reliable diagnostic tools are necessary for an effective treatment. Standard diagnostic techniques, such as culturing and zoospore induction, take several days and require taxonomic expertise, causing delayed treatment. Many advanced antibody-based assays have been developed, including an immunodiffusion test[14,15], an enzyme-linked immunosorbent assay (ELISA)[16-18], indirect immuno-fluorescence[14] and immunoblot[18-21]. These techniques have facilitated the rapid detection of *P. insidiosum* infection. Since many advantages of immunoblot have been determined, this technique has been chosen for pythiosis diagnostic tool in our hospital. Although the presented immunoblot assay has been studied previously, the efficiency of the technique as a rapid diagnostic test for human pythiosis has not been evaluated. In the present study, the appropriate strain of *P. insidiosum* and type of antigens under different culture conditions were determined. Then, the immunoblot assay with an appropriate condition was applied for diagnosing samples from all suspected cases collected in our hospital since 2002. The specificity of the assay was evaluated using immunized rabbit sera and naturally infected patients' sera with other proven fungal infections. Moreover, culturing and PCR assay were also applied to determine the reliability of the immunoblot result.

Material and Method

Microorganism

Four *Pythium insidiosum* reference strains (CBS119452, CBS119453, CBS119454, and CBS119455), isolated from humans with pythiosis in Thailand, were used for antigen preparation.

Sera

Twenty-six serum samples were obtained from patients who had presented with a clinical suspicion of pythiosis, including gangrene and thalassemia as an underlying disease. Twenty-five of the twenty-six samples were collected from patients with active infection but one sample was obtained from a pythiosis patient who had been cured a year earlier by limb amputation. Cross-reactivity of the immunoblot assay was performed using sera from ten healthy individuals and five patients with other proven fungal infections, including candidiasis, cryptococcosis, aspergillosis, penicilliosis marneffei, and histoplasmosis.

Four control rabbit antisera were prepared by immunizing white rabbits with antigens of human pathogenic fungi, including *Pythium insidiosum, Cryptococcus neoformans, Candida albicans, Penicillium marneffei,* and *Histoplasma capsulatum.* The rabbits were injected subcutaneously with a 1 ml mixture of the concentrated antigens and 1 ml of incomplete Freund adjuvant. After 2 and 4 weeks, the rabbits were immunized subcutaneously and then bled 2 weeks later.

Protein preparation

Pure cultures of *P. insidiosum* were analyzed for their protein and immunoblot profiles. Briefly, the cultures were grown in 2% Sabouraud dextrose broth (SDB) pH 6.9 at 37°C on a shaker rotating at 150 rpm for 5 days. Mycelia harvested by vacuum filtration were washed three times with sterile PBS pH 7.2 and resuspended in 1 ml of sterile PBS containing a protease inhibitor cocktail [4-(2-aminoethyl) benzene-sulfonyl fluoride (AEBSF), pepstatin A, E-64, and 1, 10-phenanthroline] (Sigma, St Louis, USA). Protein antigens were extracted from the mycelium mass using a bead beating procedure. Briefly, mycelium mass was homogenized with 500 mg of 0.4-0.6 mm diameter glass beads (Biospec, Oklahoma, USA) in a microcentrifuge tube at full speed for 30 s. The samples obtained after bead-beating were centrifuged at 12,000 *g* for 15 min and then the supernatant was collected and used as a cytoplasmic antigen. Secretory protein antigens were prepared from cell-free culture filtrate antigens and concentrated by ammonium sulfate precipitation. The proteins were suspended in PBS pH 7.2 containing a protease-inhibitor cocktail (Sigma, St Louis, USA). The protein concentration of the samples was measured by a Bio-Rad microassay procedure (Bio-Rad Laboratories, Milan, Italy). The samples were stored at 4°C until use.

To determine whether different culture media affected the secretory protein profile of *P. insidiosum*, a strain CBS119452 was cultured in different types of media including 1. home-made SDB pH 6.9 (20 g glucose and 10 g polypeptone) (Beckton Dickinson, Cockeysville, MD, USA), 2. SDB pH 6.9 (Difco Laboratories, Detroit, MI, USA), 3. SDA pH 6.9 (Scharlau Chemie S.A., Barcelona, Spain), 4. Brain-Heart infusion broth (BHIB) (Difco), 5. BHI broth (Oxoid, Basingstoke, UK) and 6. Nutrient broth (Himedia, Mumbai, India). The strain was cultured using the method described above.

SDS-PAGE

Twenty micrograms of each protein extract was solubilized in a sample buffer containing 62.5 mM Tris-HCl (pH 6.8), 5% 2-b-mercaptoethanol, 10% glycerol, 5% sodium dodecyl sulfate (SDS) and 0.01% bromphenol blue, and then boiled for 5 min. The protein extracts were loaded onto constant gradient gels (10%) and run in a Mini Protean II slab cell system (Bio-Rad Laboratories, Milan, Italy) at a constant current of 100 V for 1 h 45 min. The gels were stained for protein with 0.2% Coomassie brilliant blue R-250.

Immunoblotting

Immunoblotting was performed using the following method; one microgram of the cytoplasmic and secretory protein antigens was separated by SDS-PAGE, and then transferred to the nitrocellulose membrane (Amersham). The blot was incubated with 5% skimmed milk blocking buffer for 2 h at room temperature and then washed three times in phosphate-buffered saline (PBS) with 0.05% Tween 20. The blot was cut into strips. The strips were then incubated with each serum sample (1:100 dilution) for 1 h at 37°C before washing three times in PBS-Tween. Following incubation with horseradish peroxidase-conjugated goat anti-human IgG (1:1,000 dilution) or anti-rabbit IgG (1:3,000 dilution), the reaction was detected with 4-chloro-1-naphthol as a substrate.

Specimen processing

The specimens, including biopsy material, exudates and/or arterial tissue (Fig. 1a), were processed immediately after being received. Biopsy tissues were cut into small pieces using aseptic technique, and then cultured on Sabouraud glucose agar (SGA) pH 6.9 (Fig. 1b). The cultures were examined daily. Morphological identification based on the characteristics of sporangia was performed using a previously described method[18].

PCR amplification

For the PCR assay, specific primers ITSpy1 (5'-CTGCGGAAGGATCATTACC-3') and ITSpy2 (5'-GTCCTCGGAGTATAGATCAG-3')[18] were used to amplify the internal transcribed spacer region of *P. insidiosum*. The DNA of the specimen was extracted with the Dneasy® tissue kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instruction. PCR conditions were performed as follows: 94°C for 3 min; 35 cycles at 94°C for 45 s, 60°C for 30 s, and 72°C for 30 s; and a final extension at 72°C for 10 min. The amplicons of approximately 233-bp were analyzed by agarose gel electrophoresis, followed by ethidium bromide staining.

Results

Immunogen profile of P. insidiosum clinical isolates

To develop an immunoblot assay for the diagnosis of human pythiosis, the appropriate strains of *P. insidiosum* and types of antigens were determined. The protein- and immunogen profiles of four clinical isolates of *P. insidiosum* are shown in Fig. 2a and 2b. All four strains showed similar cytoplasmic protein profiles by SDS-PAGE and immunoblotting. Dominant protein bands of approximately 86, 68, 53 to 56, 49, 36, 34, 25, 18, and 16 kDa were observed in the Coomassie blue-stained gel (Fig. 2a, left panel). The 86, 76 and 38 kDa bands were dominant antigens on the immunoblot (Fig. 2b, left panel). In contrast to the cytoplasmic protein antigens, secretory proteins of these strains showed significant differences in both protein and immunogen profiles. Several bands ranging from 130 to 17 kDa and lower in molecular weight were observed on the SDS-PAGE gel. The 70, 45, and 42 to 35 kDa bands indicated prominent antigens in all strains (Fig. 2a, right panel). Immunodominant bands of 70

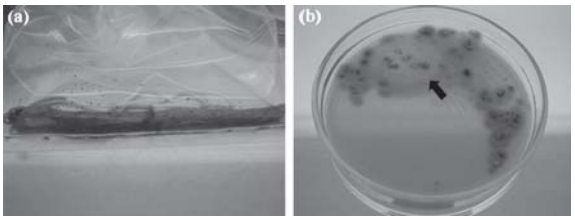

Fig. 1 Clinical specimens of pythiosis patients. (a) femur artery (b) culture of *P. insidiosum* growing from clinical specimens (arrow)

and 42 to 35 kDa were particularly prominent in all strains (Fig. 2b, right panel). However, three more bands including 28, 26 and 23 kDa were found only in strain CBS 119452 (Fig. 2b, right panel, lane 1).

Although the cytoplasmic proteins of the isolates showed several dominant bands on SDS-PAGE, only some bands were detectable on the immunoblot assay. All strains showed very similar patterns and at least five bands could be detected. On the other hand, from the secretory protein profiles of these four strains, the profile of *P. insidiosum* strain CBS 119452 provided the most proper pattern, containing many high-intensity reactive bands and well-distributing on the blot. Thus, the secretory antigen profile of strain CBS 119452 could be a good candidate for facilitating the diagnostic assay for human pythiosis.

Comparison of immunodominant antigens of P. insidiosum cultured in different culture media

The secretory protein profiles of strain CBS 119452 cultured in different culture media are shown in Fig. 3. The proteins on SDS-PAGE gel exhibited slightly different profiles among various types of culture media. Many bands ranging from 100 to 23 kDa were detected on the gel (Fig. 3a). After probing with immunized rabbit anti-*P. insidiosum* antibodies, prominent bands of 100, 70, 40 to 35, 28, 26 and 23 kDa were clearly observed in home-made and Difco SDB (Fig. 3b, lane 1 and 2). However, no 26-kDa band was detected in Scharlau SDB and Nutrient broth (Fig. 3b, lane 3 and 6). The 70-kDa band also could not be observed in BHI and Nutrient broth (Fig. 3b, lane 4 to 6). Therefore, home-made and Difco SDB were deemed to be suitable media for further antigen preparation and immunoblot analysis in the present study.

Pythiosis diagnosis using immunoblotting

Sixteen of twenty-six sera were positive to pythiosis. Eight human-pythiosis positive samples were selected to show here (Fig. 5a, lanes 3-10) comparing with positive and negative immunoblot controls probed with *P. insidiosum*-immunized and pre-immunized

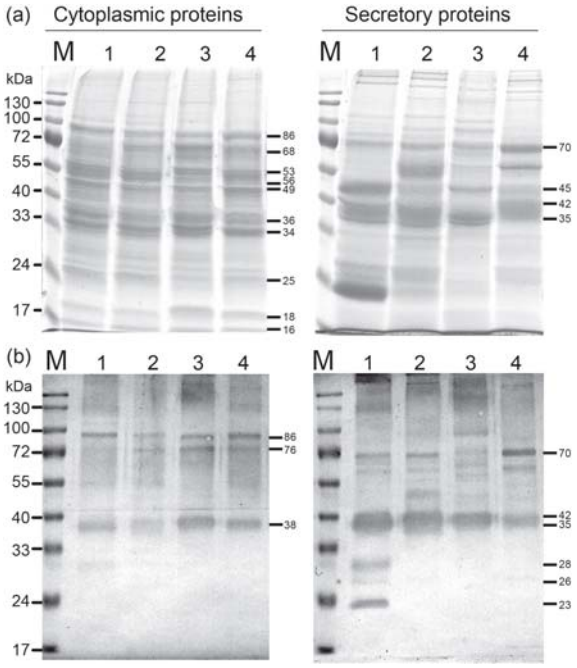

Fig. 2 Protein and immunogen profiles of four clinical isolates of *P. insidiosum*. Coomassie blue stained SDS-PAGE gel (a) and immunoblot (b), probed with *P. insidiosum*-immunized rabbit antiserum. Lanes 1-4, strains nos. CBS 119452, CBS 119453, CBS 119454 and CBS 119455, respectively. Cytoplasmic and secretory proteins are shown on the left and right panels, respectively

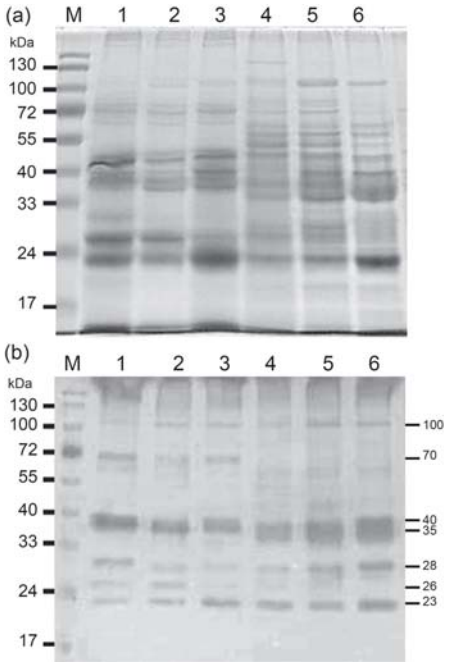

Fig. 3 Secretory protein profiles of *P. insidiosum* cultured in various types of media. Coomassie blue stained SDS-PAGE gel (a) and immunoblot probed with an immunized rabbit anti-*P. insidiosum* antibodies (b). *P. insidiosum* strain CBS119452 cultured in home-made SDB (lane 1), Difco SDB (lane 2), Scharlau SDB (lane 3), Difco BHIB (lane 4), Oxoid BHIB (lane 5) and nutrient broth (lane 6)

rabbit antiserum (Fig. 5a, lanes 1 and 2, respectively). Bands of 100, 70, 42, 40 to 35, 28, 26, and 23 kDa were found in seven samples, except one serum that showed only 70 and 40 to 35 kDa bands (Fig. 5a, lane 7). In the presented case, the serum was collected from a patient who had been cured a year earlier by limb amputation (Fig. 5a, lane 7). Serum samples from healthy subjects, *C. neoformans*-immunized rabbit antisera, and sera from cryptococcosis-, aspergillosis-, and penicilliosis marneffei patients recognized the 70-kDa antigen. No band was seen in reactions where serum samples of candidiasis and histoplasmosis patients or *Penicillium marneffei* and *Histoplasma capsulatum*-immunized rabbit sera were applied. This data suggested that the 70-kDa band observed in the controls had poor specificity. The bands of 100 and 26 kDa were reactive with all serum samples of the patients with active pythiosis, while the 42- and 23-kDa bands were found only in some cases. Interestingly, the 40 to 35-kDa antigen was reactive specifically with all sera from both treated and active pythiosis patients. Ten samples from patients with suspected pythiosis were immunoblot-negative (Table 1). In seven cases of immunoblot-positive and four cases of immunoblot-negative results, more clinical specimens of these patients were collected with agreement for alternative diagnostic tests.

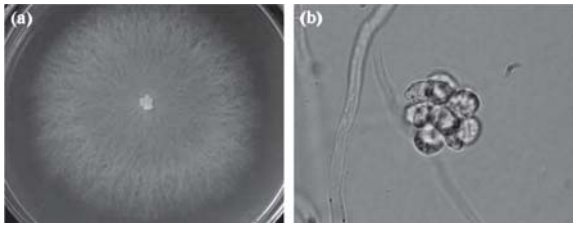

Fig. 4 Morphology of clinical isolate of *P. insidiosum*. Glabrous colony on Sabouraud's agar at 25°C (a) and zoospore production in induction medium (b), magnification x 400

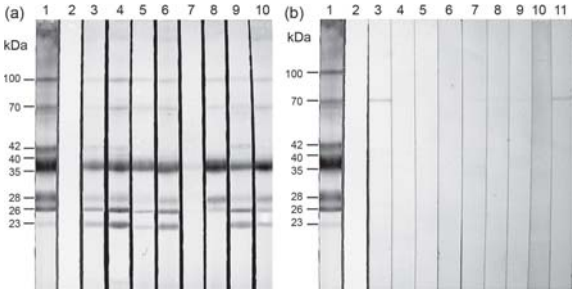

Fig. 5 Immunoblot analysis of *P. insidiosum*. Culture filtrate antigens reacting with *P. insidiosum*-immunized rabbit antiserum (lane 1 of a and b), pre-immunized rabbit antiserum (lane 2 of a and b) and pythiosis patients'sera, lane 3-10 (a). The reactivities with (b) pathogenic fungi-immunized rabbit antisera; *Cryptococcus neoformans* (lane 3), *Penicillium marneffei* (lane 4), *Histoplasma capsulatum* (lane 5) and sera of patients with other fungal infections; candidiasis (lane 6), cryptococcosis (lane 7), aspergillosis (lane 8), penicillosis marneffei (lane 9), histoplasmosis (lane 10) and pooled sera of 10 healthy individuals (lane 11)

Comparison between immunoblot, culture and PCR assay

Culture and PCR methods were performed in parallel with the immunoblot assay in order to determine the accuracy of the immunoblot. Specimens such as arteries from amputated limbs, wound exudates, pus, or tissue biopsy were collected from some patients for culturing and DNA extraction. *P. insidiosum* could be isolated only from the arteries collected from amputated limbs of the patients whose immunoblots were positive (Table 1). All isolates showed a white cream glabrous colony with broad, sparsely septate hyaline hyphae and produced filamentous sporangia and motile biflagellate zoospores in induction medium (Fig. 4a, 4b). The organism grew well within a few days at both 37°C and room temperature. In addition, PCR results were well consistent with immunoblot and culturing (Table 1). However, it was likely that an appropriate sample collection was also important for pythiosis identification by PCR and culturing since there were inconsistencies in some cases such as sample no. 2 in Table 1 which was immunoblot-positive but negative for both culture and PCR. In the cases of the immunoblot-negative result, the authors found that the PCR and culture findings were also negative in all cases. Therefore, the present study revealed that the immunoblot assay is easy, highly specific and very useful for being a rapid pythiosis diagnostic test in our hospital.

Discussion

Immunoblot analysis for the detection and identification of *P. insidiosum* has been studied since 1992. The assay has been studied in order to demonstrate the immune response of infected animals and humans to *P. insidiosum*. The specific immunoblot profile of *P. insidiosum* infection has been characterized

Table 1. Diagnostic results of immunoblot, culture and PCR assay from twenty-six suspected pythiosis cases

Sample	Specimen	Immunoblot	Culture	PCR
1	serum and pus	Pos	Neg	Pos
2	serum, pus and vessel	Pos	Neg	Neg
3	serum	Pos	N/D	N/D
4	serum	Pos	N/D	N/D
5	serum and artery	Pos	Pos	Pos
6	serum	Pos	N/D	N/D
7	serum, wound exudates and artery	Pos	Pos	Pos
8	serum and artery	Pos	Pos	Pos
9	serum	Pos	N/D	N/D
10	serum	Pos	N/D	N/D
11	serum	Pos	N/D	N/D
12	serum	Pos	N/D	N/D
13	serum	Pos	N/D	N/D
14	serum and artery	Pos	Pos	Pos
15	serum	Pos	N/D	N/D
16	serum and tissue biopsy	Pos	Neg	N/D
17	serum and occlusion	Neg	Neg	Neg
18	serum and pus	Neg	Neg	Neg
19	serum and vessel	Neg	Neg	Neg
20	serum, vessel and formalinized tissue	Neg	Neg	Neg
21	serum	Neg	N/D	N/D
22	serum	Neg	N/D	N/D
23	serum	Neg	N/D	N/D
24	serum	Neg	N/D	N/D
25	serum	Neg	N/D	N/D
26	serum	Neg	N/D	N/D

Pos = positive, Neg = negative, N/D = not done

in many hosts such as horse, rabbit, cattle, and human. Immunodominant antigens of *P. insidiosum* have been determined. The immunogens of 80-, 33.5, 32-, 30- and 28-kDa bands commonly reacted with sera from horses infected with *P. insidiosum*[19,20]. A 74-kDa immunodominant antigen was recognized by sera from pythiosis patients[21]. In the present study, the bands of 70- and 40 to 35-kDa were observed in all pythiosis patients. However, sera from healthy subjects and other fungal-infected patients also recognized the 70-kDa band. Hence, the 70-kDa antigen found in the present study was apparently a common fungal antigen. The band of 40 to 35 kDa that reacted specifically with pythiosis patients' sera was a potential indicator of *P. insidiosum* infection. Interestingly, the immunoglobulin G reacting to the 40 to 35-kDa band was still found in the 1-year treated patient, suggesting that this protein may be an important protective immunogen in humans. In addition, the immunoblot profile of 100-, 42-, 28-, 26- and 23-kDa bands, which was found to react with sera from active pythiosis patients, may be useful for indicating a pythiosis infection. Moreover, none of these bands could be detected by the treated patient's serum (Fig 5, lane 7). Thus, the profile might be useful both for diagnosis and for monitoring the disease and treatment.

The 40 to 35-kDa protein was an important immunodominant antigen found in both clinical and environmental strains. It was a highly specific antigen for *P. insidiosum* since it was recognized only in this organism but not in its closely related species, *P. grandisporangium and P. cystogenes*[10]. Since pythiosis cases have been increasing and aquatic agricultural areas are known to be a high-risk factor for *P. insidiosum* infection, the development of effective diagnostic and screening methods is imperative. This antigen might be useful for developing a pythiosis diagnostic test and a *P. insidiosum* screening test to be applied in field work in the endemic areas of human and animal pythiosis.

Immunized rabbits' immune response to *P. insidiosum* displayed a unique profile similar to that of naturally infected humans. This finding supports previous studies suggesting that rabbits could be a good model to study the pathogenesis and immune response to *P. insidiosum* infection[20,22]. Moreover, hematological disorders, especially thalassemia, have been reported as a common underlying disease found in pythiosis patients. In order to study the immune response of patients with hematological disorders to *P. insidiosum* infection, an animal model such as thalassemic mice may provide an alternative way to further investigate this aspect.

Several fungi have morphological similarities to the filamentous structures of *P. insidiosum*. Histological examination might prove difficult when differentiating pythiosis from those found in cases of oomycosis caused by *Lagenidium giganteum,* and zygomycosis caused by fungal species of the orders Mucorales and Entomophthorales[23]. Basically, the isolation of *P. insidiosum* is the ultimate basis for the diagnosis of pythiosis. The identification of *P. insidiosum* in the laboratory is definitely based on its asexual zoosporangia, sexual oogonia, and hyphae-like morphological features. However, culturing and morphological identification have several limitations, mainly that they are time-consuming and require expertise. Furthermore, an appropriate biopsy specimen is very important for the success of fungal isolation. In the present study, the arterial tissue containing the occlusion, which was isolated from an infected organ, was proved to be highly positive for culturing, while pus and skin biopsy obtained from infected wounds might be unsuitable. It is necessary for the surgeon to have a strong clinical suspicion of pythiosis to obtain the appropriate specimens. Since the proper sample as arterial tissue could be provided after amputation only, the method may not be suitable for early diagnosis. To overcome the limitations of the method, serodiagnostic techniques, including the immunoblot assay, may provide a fast and convenient alternative for early diagnosis of pythiosis.

A PCR-based assay for pythiosis diagnosis was developed in our laboratory to diagnose and identify human *P. insidiosum* isolates. Vanittanakom et al. developed primers that amplified 233 base pairs in the internal transcribed spacer (ITS) regions[18]. The PCR provides excellent specificity for diagnosis of pythiosis since it does not amplify DNA from other human pathogenic fungi and bacteria (unpublished data). The PCR assay was a good method for identifying *P. insidiosum* from a broad range of specimens, including frozen specimen, ethanol-fixed tissue[24] and fresh specimens such as arterial tissue, tissue biopsy, pus, and drained secretions from infected wounds. However, in the present study, it should be noted that the assay was often negative with DNA extracted from skin biopsies. Perhaps this specimen was not appropriate. As in culturing, the success of PCR depends on the presence of hyphae in the tissue evaluated. Thus, when the concentration of *P. insidiosum* DNA is low due to either DNA loss during extraction process or limited number of hyphae obtained from original clinical samples, the presented PCR may provide false negative results. In such cases, nested PCR could be an alternative tool for *P. insidiosum* detection. Yet, although nested PCR has been demonstrated as an effective assay for identification of *P. insidiosum*[25], some of presented clinical isolates could not be amplified using primers described in the literature. Unlike PCR and culturing, the immunoblot assay for pythiosis diagnosis does not need any special requirements for sample collection.

In conclusion, the present study demonstrated the usefulness of the immunoblot assay as a rapid detection and diagnostic tool for *P. insidiosum* infection. The present results provided new information on the advantages of the immunoblot assay, especially regarding its non-invasive nature and ease of use when compared to standard methods such as culturing and to high-sensitivity assays such as PCR. The immunoblot assay is the most rapid and sensitive diagnostic tool recommended for human pythiosis. The assay has been proven effective, especially for early diagnosis after admitting patients to the hospital for appropriate treatment. Combining the immunoblot profile with the results from PCR and culturing provides reliable results and an accurate diagnosis, which is important for treating and monitoring the disease.

Acknowledgements

This work was supported in part by grants from the Royal Golden Jubilee PhD Program, the Thailand Research Fund (for Supabandhu, J) and from the Faculty of Medicine and Graduate School, Chiang Mai University, Chiang Mai, Thailand.

References

1. Sathapatayavongs B, Leelachaikul P, Prachaktam R, Atichartakarn V, Sriphojanart S, Trairatvorakul P, et al. Human pythiosis associated with thalassemia hemoglobinopathy syndrome. J

Infect Dis 1989; 159: 274-80.
2. Prasertwitayakij N, Louthrenoo W, Kasitanon N, Thamprasert K, Vanittanakom N. Human pythiosis, a rare cause of arteritis: case report and literature review. Semin Arthritis Rheum 2003; 33: 204-14.
3. Wanachiwanawin W, Thianprasit M, Fucharoen S, Chaiprasert A, Sudasna N, Ayudhya N, et al. Fatal arteritis due to Pythium insidiosum infection in patients with thalassaemia. Trans R Soc Trop Med Hyg 1993; 87: 296-8.
4. Triscott JA, Weedon D, Cabana E. Human subcutaneous pythiosis. J Cutan Pathol 1993; 20: 267-71.
5. Krajaejun T, Pracharktam R, Wongwaisayawan S, Rochanawutinon M, Kunakorn M, Kunavisarut S. Ocular pythiosis: is it under-diagnosed? Am J Ophthalmol 2004; 137: 370-2.
6. Virgile R, Perry HD, Pardanani B, Szabo K, Rahn EK, Stone J, et al. Human infectious corneal ulcer caused by Pythium insidiosum. Cornea 1993; 12: 81-3.
7. Imwidthaya P. Human pythiosis in Thailand. Postgrad Med J 1994; 70: 558-60.
8. Wanachiwanawin W. Infections in E-beta thalassemia. J Pediatr Hematol Oncol 2000; 22: 581-7.
9. Kunavisarut S, Nimvorapan T, Methasiri S. Pythium corneal ulcer in Ramathibodi Hospital. J Med Assoc Thai 2003; 86: 338-42.
10. Supabandhu J, Fisher MC, Mendoza L, Vanittanakom N. Isolation and identification of the human pathogen Pythium insidiosum from environmental samples collected in Thai agricultural areas. Med Mycol 2008; 46: 41-52.
11. Mendoza L, Hernandez F, Ajello L. Life cycle of the human and animal oomycete pathogen Pythium insidiosum. J Clin Microbiol 1993; 31: 2967-73.
12. Mendoza L, Newton JC. Immunology and immunotherapy of the infections caused by Pythium insidiosum. Med Mycol 2005; 43: 477-86.
13. Krajaejun T, Sathapatayavongs B, Pracharktam R, Nitiyanant P, Leelachaikul P, Wanachiwanawin W, et al. Clinical and epidemiological analyses of human pythiosis in Thailand. Clin Infect Dis 2006; 43: 569-76.
14. Mendoza L, Kaufman L, Standard P. Antigenic relationship between the animal and human pathogen Pythium insidiosum and nonpathogenic Pythium species. J Clin Microbiol 1987; 25: 2159-62.
15. Pracharktam R, Changtrakool P, Sathapatayavongs B, Jayanetra P, Ajello L. Immunodiffusion test for diagnosis and monitoring of human pythiosis insidiosi. J Clin Microbiol 1991; 29: 2661-2.
16. Krajaejun T, Kunakorn M, Niemhom S, Chongtrakool P, Pracharktam R. Development and evaluation of an in-house enzyme-linked immunosorbent assay for early diagnosis and monitoring of human pythiosis. Clin Diagn Lab Immunol 2002; 9: 378-82.
17. Mendoza L, Kaufman L, Mandy W, Glass R. Serodiagnosis of human and animal pythiosis using an enzyme-linked immunosorbent assay. Clin Diagn Lab Immunol 1997; 4: 715-8.
18. Vanittanakom N, Supabandhu J, Khamwan C, Praparattanapan J, Thirach S, Prasertwitayakij N, et al. Identification of emerging human-pathogenic Pythium insidiosum by serological and molecular assay-based methods. J Clin Microbiol 2004; 42: 3970-4.
19. Mendoza L, Nicholson V, Prescott JF. Immunoblot analysis of the humoral immune response to Pythium insidiosum in horses with pythiosis. J Clin Microbiol 1992; 30: 2980-3.
20. Leal AT, Santurio JM, Leal AB, Catto JB, Flores EF, Lubeck I, et al. Characterization of the specificity of the humoral response to Pythium insidiosum antigens. J de Mycologie M□dicale 2005; 15: 63-8.
21. Krajaejun T, Kunakorn M, Pracharktam R, Chongtrakool P, Sathapatayavongs B, Chaiprasert A, et al. Identification of a novel 74-kiloDalton immunodominant antigen of Pythium insidiosum recognized by sera from human patients with pythiosis. J Clin Microbiol 2006; 44: 1674-80.
22. Santurio JM, Leal AT, Leal AB, Festugatto R, Lubeck I, Sallis ES, et al. Three types of immunotherapics against pythiosis insidiosi developed and evaluated. Vaccine 2003; 21: 2535-40.
23. Grooters AM, Hodgin EC, Bauer RW, Detrisac CJ, Znajda NR, Thomas RC. Clinicopathologic findings associated with Lagenidium sp. infection in 6 dogs: initial description of an emerging oomycosis. J Vet Intern Med 2003; 17: 637-46.
24. Znajda NR, Grooters AM, Marsella R. PCR-based detection of Pythium and Lagendium DNA in frozen and ethanol-fixed animal tissues. Vet Dermatol 2002; 13: 187-94.
25. Grooters AM, Gee MK. Development of a nested polymerase chain reaction assay for the detection and identification of Pythium insidiosum. J Vet Intern Med 2002; 16: 147-52.

การประยุกต์ใช้วิธีอิมมูโนบลอทสำหรับการตรวจวินิจฉัยอย่างรวดเร็วของโรคพิทิโอสิสในมนุษย์

จิดาภา สุภาพันธุ์, ปราโมทย์ วณิตย์ธนาคม, กำพล เลาหเพ็ญแสง, นงนุช วณิตย์ธนาคม

วัตถุประสงค์: *โรคพิทิโอสิสเป็นโรคติดเชื้อที่มีอันตรายถึงแก่ชีวิตได้มีรายงานในโรงพยาบาลมหาราชนครเชียงใหม่ตั้งแต่ปี พ.ศ. 2544 การวินิจฉัยโรคที่ล่าช้าทั้งนี้อาจเนื่องมาจากการความไม่เหมาะสมของสิ่งส่งตรวจและการตรวจหาเชื้อ สาเหตุต้องใช้เวลานานจึงเป็นสาเหตุทำให้การรักษาเป็นไปอย่างล่าช้าเช่นกัน ส่งผลให้มีอัตราการเสียชีวิตค่อนข้างสูง ดังนั้นเพื่อแก้ไขปัญหาดังกล่าวจึงได้นำวิธีอิมมูโนบลอทที่เคยได้พัฒนามาประเมินประสิทธิภาพและนำมาใช้จริง ในการตรวจวินิจฉัยโรคพิทิโอสิสในมนุษย์ ในโรงพยาบาลนี้*

วัสดุและวิธีการ: *การประเมินวิธีอิมมูโนบลอททำได้โดยใช้ซีรัมของผู้ป่วยที่ผ่านการตรวจพิสูจน์เชื้อเป็นที่เรียบร้อย โดยในกลุ่มควบคุมได้ใช้ซีรัมของผู้ป่วยติดเชื้อรา, ซีรัมจากคนปกติ รวมทั้ง ซีรัมที่ได้จากการฉีดกระต่ายด้วยเชื้อ Cryptococcus neoformans, Penicillium marneffei และ Histoplasma capsulatum จากนั้นได้ใช้วิธีดังกล่าวในการทดสอบกับซีรัมของผู้ป่วยที่สงสัยว่าจะเป็นโรคพิทิโอสิสจำนวน 26 ราย นอกจากนี้ยังใช้วิธีการเพาะเชื้อและวิธีปฏิกิริยาลูกโซ่พอลิเมอเรสในการตรวจวินิจฉัยร่วมกับวิธีอิมมูโนบลอทในบางรายที่มีความเหมาะสมเพื่อทดสอบความถูกต้องของวิธี อิมมูโนบลอท*

ผลการศึกษา: *วิธีอิมมูโนบลอทที่ใช้ไม่ทำปฏิกิริยากับซีรัมจากกลุ่มควบคุมที่ใช้ในการทดลองครั้งนี้แต่อย่างใด โรคพิทิโอสิสสามารถตรวจวินิจฉัยแยกโรคจากโรคติดเชื้อราชนิดอื่นที่มีอาการคล้ายคลึงกัน จากการทดลองสามารถแยกผู้ป่วยโรคพิทิโอสิสได้ 16 รายจากผู้ป่วยที่สงสัยทั้งหมด 26 ราย ในรายที่ให้ผลบวกได้แสดงแบบแผนการทำปฏิกิริยาเหมือนกันกับในซีรัมของกระต่ายที่ถูกฉีดกระตุ้นด้วยเชื้อ Pythium insidiosum การศึกษาครั้งนี้แสดงให้เห็นว่าโปรตีนขนาด 40-35 กิโลดาลตันจำเพาะต่อโรคพิทิโอสิสเนื่องจากเกิดปฏิกิริยากับซีรัมของผู้ป่วยพิทิโอสิสทุกราย นอกจากนี้ผลการทดลองจากวิธีเพาะเชื้อและวิธีปฏิกิริยาลูกโซ่พอลิเมอเรส มีความสอดคล้องกันกับวิธีอิมมูโนบลอทที่ใช้*

สรุป: *วิธีอิมมูโนบลอทที่ได้พัฒนานี้มีความจำเพาะต่อการติดเชื้อ P. insidiosum และเหมาะสำหรับใช้เป็นเครื่องมือในการตรวจวินิจฉัยโรคพิทิโอสิสได้อย่างมีประสิทธิภาพ*