EVALUATION OF AN IN-HOUSE IMMUNOPEROXIDASE STAINING ASSAY FOR HISTODIAGNOSIS OF HUMAN PYTHIOSIS

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Abstract. Pythiosis, a life-threatening infectious disease of humans and animals in tropical and subtropical countries, is caused by the fungus-like organism *Pythium insidiosum*. As diagnosis of pythiosis is difficult, delayed diagnosis of pythiosis leads to poor prognosis. We developed an immunoperoxidase staining assay using rabbit anti-*P. insidiosum* antibodies to detect *P. insidiosum* directly in infected tissues of 19 patients with vascular (n = 11), ocular (n = 7) or cutaneous (n = 1) pythiosis. Tissue sections from 31 patients with various fungal infections were included as controls. Tissue sections from all pythiosis patients and 2 patients with *Fusarium* infections were stained positive, whereas the other 29 control sections were stained negative. Sensitivity and specificity of the assay was 100% and 94%, respectively. Based on the prevalence of human pythiosis (2%), calculated positive predictive value and negative predictive value was 24% and 100%, respectively. Thus, the diagnostic value of this assay is for ruling out pythiosis. The assay requires routine laboratory equipments and can easily be performed by pathologists in rural hospitals where the disease is more prevalent.

INTRODUCTION

Pythiosis is a life-threatening infectious disease of humans and animals (viz, horses, dogs, cats, cattle) living in tropical and subtropical countries (Mendoza *et al*, 1996; Thianprasit *et al*, 1996; Kaufman, 1998; Bosco Sde *et al*, 2005; Rivierre *et al*, 2005; Krajaejun *et al*, 2006b). The causative agent is the fungus-like organism *Pythium insidiosum*, which is a member of Class Oomycetes and Phylum Pseudofungi in the Kingdom Chromista (Stramenopila) (De Cock *et al*, 1987; Mendoza *et al*, 1996; Thianprasit *et al*, 1996; Kaufman, 1998). *P. insidiosum* inhabits swampy areas, where it is present in 2 stages, namely, mycelium and zoospore (De Cock *et al*, 1987; Mendoza *et al*, 1993). Zoospore is an infective unit that attaches, germinates, and causes pathology in host tissue (Mendoza *et al*, 1993).

Human pythiosis is endemic in Thailand

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(Krajaejun *et al*, 2006b). Thalassemia, a major genetic disorder of the Thai population, and agricultural-related work are prominent predisposing factors for human pythiosis (Sathapatayavongs *et al*, 1989; Krajaejun *et al*, 2006b). A number of forms of human pythiosis have been described (Krajaejun *et al*, 2006b): (i) vascular pythiosis affecting arteries leading to arterial occlusion and aneurysm, (ii) ocular pythiosis affecting cornea leading to keratitis or corneal ulcer, (iii) cutaneous/subcutaneous pythiosis affecting limbs leading to a granulomatous and ulcerating lesion, and (iv) disseminated pythiosis affecting internal organs. Vascular (59% of cases) and ocular (33%) infections are the most common forms of pythiosis. The main treatment option for pythiosis is radical surgery, which should be performed urgently to limit disease progression. Most patients with vascular pythiosis undergo leg amputation, while most patients with ocular pythiosis undergo eye removal. Many pythiosis patients die as a result of advanced infection.

Early and correct diagnosis can lead to early and proper treatment and better prognosis. Culture identification and serological methods (*viz*, immunodiffusion test, enzyme-linked immunosorbent assay, Western blot, immunochromatographic test and hemagglutination test) are now available for diagnosis of pythiosis (Mendoza *et al*, 1986, 1997; Mendoza and Prendas, 1988; Chaiprasert *et al*, 1990; Pracharktam *et al*, 1991; Krajaejun *et al*, 2002, 2006a, 2009; Jindayok *et al*, 2009). Nevertheless, use of these methods is still problematic in some circumstances; for example, culture identification often fails to isolate *P. insidiosum* from clinical specimens, and serodiagnostic tests have high rates of false negative results for sera of ocular pythiosis patients (Jindayok *et al*, 2009; Krajaejun *et al*, 2009). Additionally, paraffin-embedded tissue sometimes is the only available specimen for diagnosis and it is not applicable for testing with these methods. Recently, polymerase chain reaction (PCR) and DNA sequence analysis were developed for detection of *P. insidiosum* (Badenoch *et al*, 2001; Grooters and Gee, 2002; Znajda *et al*, 2002; Vanittanakom *et al*, 2004). However, use of these molecular techniques is limited because of their complicated procedures and costly reagents and equipments. Therefore, finding a diagnostic modality for diagnosis of pythiosis is still an important healthcare goal.

In pathology laboratories, several non-specific staining techniques, viz, Grocott's methenamine silver (GMS) and Periodic acid-Schiff (PAS) have been routinely used to demonstrate fungus in infected tissues. *P. insidiosum* can be stained by GMS and PAS (Krajaejun *et al*, 2006b), but the pathogen has no pathognomonic microscopic feature. Interpretation of GMS and PAS stains is further complicated by the fact that *P. insidiosum* has similar morphology (broad and branching hyphae) to some filamentous fungi (zygomycetes, *Aspergillus* spp, and *Fusarium* spp), which can lead to a misdiagnosis (Mendoza *et al*, 2004). Brown *et al* (1988) described an immunohistochemical method for diagnosis of pythiosis in horse. As their assay was evaluated against a limited number of control tissues with other fungal infections, this raises a question regarding detection specificity. In the present study, we developed an immunoperoxidase staining (IPS) assay using rabbit anti-*P. insidiosum* antibodies to directly detect *P. insidiosum* in infected tissues of human patients with pythiosis. We included control tissues from patients with a broad range of fungal infections for assay evaluation.

MATERIALS AND METHODS

P. insidiosum antigen

P. insidiosum strain CBS673.85 isolated

from a Thai patient with cutaneous pythiosis was used to prepare *P. insidiosum* antigen. The microorganism was subcultured on Sabouraud dextrose agar and incubated at 37ºC for 3 days. Several small blocks of mycelium were transferred to Sabouraud dextrose broth and shaken (150 rpm) at 37ºC for 1 week. Merthiolate was added to the culture at a final concentration of 0.02% (w/v). The culture was filtered through a Durapore membrane filter (0.22 µm pore size) and culture filtrate broth was concentrated to ~20-fold using an Amicron® 8400 apparatus with a 10,000-nominal molecular weight limit filter (Millipore, Bedford, MA). The concentrated broth was referred to as culture filtrate antigen (CFA), and was stored at -20ºC until use.

Rabbit anti-*P. insidiosum* serum

Rabbit anti-*P. insidiosum* serum was prepared by a modified method of Mendoza *et al* (1987). In brief, an adult female New Zealand White rabbit was injected intramuscularly with 1 ml of a 1:1 mixture of CFA and Freund incomplete adjuvant (Sigma) on Days 1, 2, and 3. A 0.5-ml aliquot of CFA then was injected intravenously on Days 7, 14, and 21. On Day 30, the rabbit was bled, and serum was tested for immunoreactivity using an immunodiffusion test against CFA (Pracharktam *et al*, 1991). Serum was stored at -20ºC until use.

Tissue samples

Paraffin-embedded *P. insidiosum*-infected tissue (arterial, cutaneous or corneal tissue) from 19 patients with human pythiosis (11 vascular, 7 ocular and 1 cutaneous/subcutaneous) were recruited for assay evaluation (Table 1). All pythiosis patients were diagnosed based on culture identification and induction of zoospores. Additional paraffin-embedded tissue blocks from 31 culture-proven cases of various fungal infections (7 *Candida albicans*, 6 *Aspergillus fumigatus*, 5 *Aspergillus flavus*, 4 *Fusarium* spp, 2 *Mucor* spp, and one each of *Rhizomucor* sp, *Conidiobolus* sp, *Candida parapsilosis*, *Candida tropicalis*, *Trichosporon cutaneum*, *Drechslera* sp and *Pneumocystis jirovecii*) served as controls (Table 1). Each paraffin-embedded tissue block was cut to thin slices (4-µm thickness) using a microtome. Each tissue slice was placed on a glass slide, generating tissue sections for GMS or immunoperoxidase staining.

GMS and immunoperoxidase stain

Tissue sections from all patients were stained with GMS (Grocott, 1955). A fungal organism, if present in the tissue, was sharply delineated in black when visualized under a light microscope. Immunoperoxidase staining procedure was modified from the method routinely used in the Anatomical Pathology Unit, Department of Pathology, Ramathibodi Hospital. Firstly, tissue sections were deparaffinized in xylene, rehydrated in ethanol, and washed with phosphate-buffered saline (PBS; pH 7.4). Tissue sections were treated with TE buffer (pH 9.0) in water bath at 95ºC for 40 minutes. To inactivate endogenous peroxidases, deparaffinized sections were treated with 3% H_2O_2 in methanol for 30 minutes. Non-specific binding was inhibited by incubating tissue sections with 3% horse serum albumin in PBS (blocking buffer) for 20 minutes. Tissue sections were incubated with 200 µl of rabbit anti-*P. insidiosum* serum (1:16,000 in blocking buffer) in a moist chamber at room temperature for 1 hour, and then were washed 2 times with PBS for 5 minutes. Tissue sections were subsequently incubated with 200 µl of undiluted mouse anti-rabbit antibodies conjugated with horseradish-peroxidase (EnVision+System-Labeled Polymer-HRP, Dako) for 30 minutes. After washing as described above, 1 ml of 0.1% (w/v) diaminobenzidine (Sigma) in Tris buffer (pH 7.4) was added to the tissue sections for color development. Tissue sections

Table 1
Tissue sections used in this study.

Code	Fungal identity (culture proven)	Organ/Tissue[a]	GMS[b]	IPS[c]
P1	*Pythium insidiosum*	Artery	+	+
P2	*Pythium insidiosum*	Artery	+	+
P3	*Pythium insidiosum*	Artery	+	+
P4	*Pythium insidiosum*	Artery	+	+
P5	*Pythium insidiosum*	Artery	+	+
P6	*Pythium insidiosum*	Artery	+	+
P7	*Pythium insidiosum*	Artery	+	+
P8	*Pythium insidiosum*	Artery	+	+
P9	*Pythium insidiosum*	Artery	+	+
P10	*Pythium insidiosum*	Artery	+	+
P11	*Pythium insidiosum*	Artery	+	+
P12	*Pythium insidiosum*	Eye	+	+
P13	*Pythium insidiosum*	Eye	+	+
P14	*Pythium insidiosum*	Eye	+	+
P15	*Pythium insidiosum*	Eye	+	+
P16	*Pythium insidiosum*	Eye	+	+
P17	*Pythium insidiosum*	Eye	+	+
P18	*Pythium insidiosum*	Eye	+	+
P19	*Pythium insidiosum*	Cutaneous	+	+
C1	*Rhizomucor* sp	Sinonasal	+	-
C2	*Mucor* sp	Sinonasal	+	-
C3	*Mucor* sp	Liver	+	-
C4	*Candida parapsilosis*	Stomah	+	-
C5	*Candida tropicalis*	Lung	+	-
C6	*Candida albicans*	Lung	+	-
C7	*Candida albicans*	Diaphragm	+	-
C8	*Candida albicans*	Lung	+	-
C9	*Candida albicans*	Lung	+	-
C10	*Candida albicans*	Lung	+	-
C11	*Candida albicans*	Heart	+	-
C12	*Candida albicans*	Cornea	+	-
C13	*Aspergillus fumigatus*	Trachea	+	-
C14	*Aspergillus fumigatus*	Trachea	+	-
C15	*Aspergillus fumigatus*	Intestine	+	-
C16	*Aspergillus fumigatus*	Kidney	+	-
C17	*Aspergillus fumigatus*	Lung	+	-
C18	*Aspergillus fumigatus*	Lung	+	-
C19	*Aspergillus flavus*	Lung	+	-
C20	*Aspergillus flavus*	Lung	+	-
C21	*Aspergillus flavus*	Colon	+	-
C22	*Aspergillus flavus*	Sinonasal	+	-
C23	*Aspergillus flavus*	Cornea	+	-
C24	*Trichosporon cutaneum*	Lung	+	-
C25	*Drechslera* sp	Sinonasal	+	-
C26	*Pneumocystis jiroveci*	Lung	+	-
C27	*Fusarium* sp	Cornea	+	-
C28	*Fusarium* sp	Cornea	+	-
C29	*Fusarium* sp	Cutaneous	+	+
C30	*Fusarium* sp	Cutaneous	+	+
C31	*Conidiobolus* sp	Mediastinum	+	+

[a] an infected organ or tissue from which sections were taken
[b] GMS, Grocott's methenamine silver stain
[c] IPS, immunoperoxidase stain

were counterstained with hematoxylin for 5 minutes to produce distinctly brown-stained fungal elements when visualized under a light microscope.

RESULTS

Anti-*P. insidiosum* serum from a rabbit immunized against CFA was used to stain *P. insidiosum* in all tissue sections. Rabbit anti-*P. insidiosum* antibodies (primary antibodies) bound to fungal cell wall proteins. Subsequently, mouse anti-rabbit antibody conjugated with peroxidase (secondary antibody) was used to detect the primary antibody. Diaminobenzidine was used as substrate leaving brownish product precipitated on the fungal surface. The presence of brownish-stained hyphae (Fig 1B) under a light microscope was considered positive, while the absence of brownish-stained hyphae was negative (Fig 1D).

To optimize the IPS assay, 2-fold dilutions of the rabbit anti-*P. insidiosum* serum, ranging from 1:1,000 to 1:64,000, were used to stain tissue sections from a patient with pythiosis and a patient with aspergillosis (control). A dilution of 1:16,000 was selected as the cutoff point because it demonstrated a marked brownish stain of *P. insidiosum* with minimal background in the tissue section (Fig 1B), whereas the fungus in the control section was unstained (Fig 1D).

Tissue sections from all patients with pythiosis (n = 19) and patients with other fungal infections (n = 31) (Table 1) were examined with both GMS and IPS staining

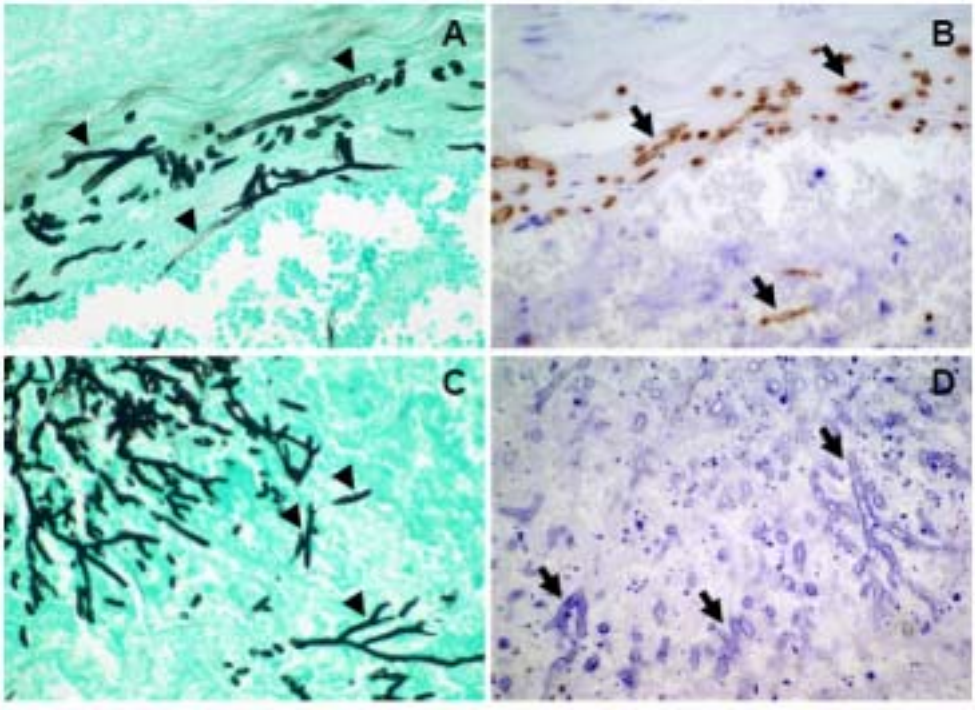

Fig 1–Histological examination of tissue sections from a patient with pythiosis (A and B), and a patient with aspergillosis (control) (C and D). A and C, arrow heads indicate non-specific GMS staining of fungal elements; B, positive IPS assay result showing brownish-stained fungal elements (*P. insidiosum*) (arrows); D, negative IPS assay result showing fungal outlines of *Aspergillus* sp (arrows) without brownish stain.

methods. GMS stain confirmed the presence of fungal elements in each tissue section (Fig 1A and 1C). Tissue sections from all patients with pythiosis and 2 control tissue sections from patients with *Fusarium* infection were read positive (presence of immunostained fungal elements; Fig 1B), whereas the other 29 control tissue sections were read negative (absence of immunostained fungal elements; Fig 1D). Sensitivity and specificity of the IPS assay was 100% and 94%, respectively.

To calculate positive (PPV) and negative (NPV) predictive values of the IPS assay, prevalence of pythiosis, detection sensitivity (100%) and detection specificity (94%) were used as in the statistical equation described elsewhere (Altman and Bland, 1994). At the Anatomical Pathology Unit, Ramathibodi Hospital during year 2008, the prevalence of pythiosis in all fungal infections (n = 152) was 2%. Thus, the PPV and NPV of the IPS assay was 24% and 100%, respectively.

DISCUSSION

Brown *et al* (1988) developed an immunperoxidase assay for histodiagnosis of pythiosis in horses. The assay correctly stained *P. insidiosum* in all tissue sections from 7 horses with pythiosis, but failed to stain several control sections from horses with *Conidiobolus* sp, *Basidiobolus* sp and zygomycete infections. However, because the assay was evaluated against only horse tissues with *P. insidiosum* infection and a limit number of control tissues, information on the sensitivity and specificity of this assay for detecting *P. insidiosum*, especially in human samples, was lacking. Therefore, we developed and evaluated an in-house IPS assay for detection of *P. insidiosum* in tissues of humans with pythiosis. Although the same immunochemical technique was applied, the assay of Brown *et al* (1988) and the present study used different antigen sources for generation of rabbit anti-*P. insidiosum* serum. Brown *et al* (1988) used crude antigen extracted from *P. insidiosum* hyphae. The present study used CFA (secreted *P. insidiosum* antigen in culture broth) because it is easier to prepare, is highly immunogenic and has been used to develop several reliable serodiagnostic tests for pythiosis (Mendoza *et al*, 1986; Pracharktam *et al*, 1991; Jindayok *et al*, 2009; Krajaejun *et al*, 2009).

The IPS assay was evaluated for its detection sensitivity against tissue sections from patients with various forms of pythiosis (P1-19; Table 1). All sections showed clear positive results, indicating that the assay has high detection sensitivity (100%). Discrimination of *P. insidiosum* infection from other fungal infections is crucial for selecting proper treatment, as well as for predicting prognosis. The assay was therefore evaluated against 31 tissue sections from patients with various fungal infections (Table 1; C1-31). All control sections, except for 2 sections from patients with *Fusarium* infection, were clearly read negative. It should be noted that IPS assay did not stain all infected tissues (C1-3, C13-23 and C31) with zygomycetes and *Aspergillus* spp (these fungi share microscopic morphology to *P. insidiosum*), indicating that the assay had a good discrimination power for these fungi. However, among 4 control sections from patients with *Fusarium* infection (C27-30), 2 each were stained negative (C27 and C28) and positive (C29 and C30), indicating that some strains or species of *Fusarium* spp cross-reacted with the rabbit anti-*P. insidiosum* antibodies. Likewise, the immunochemical assay of Brown *et al* (1988) failed to stain *Conidiobolus* sp in horse tissues whereas Grooter *et al* (2002) later reported that the assay showed some cross-reactive staining when used to evaluate tissues from dogs with conidiobolomycosis. In the future work, use of polyclonal or monoclonal anti-

body generated from a purified specific immunoreactive cell wall antigen of *P. insidiosum* should improve the specificity of the IPS assay.

Cross-reactivity to other fungi can compromise the use of IPS assay for making a definite diagnosis of pythiosis. Because pythiosis has some clinical features that mimic other mycoses, the high detection sensitivity (100%) made this IPS assay useful for screening *P. insidosum* infection in suspected case of pythiosis. In addition, statistical analysis suggested that diagnostic value of the IPS assay is for ruling out pythiosis because the assay had a very high NPV (100%). It is very important to rule out a diagnosis of pythiosis from other mycoses as treatment and prognosis are different. There is no effective noninvasive treatment for pythiosis, while many antimicrobial agents are available for successful treatment of other mycoses. Because of limited detection specificity and PPV, a positive IPS result can only suggest pythiosis and further diagnostic investigations, viz, culture identification, PCR and DNA sequence analysis are required.

In summary, an in-house IPS assay was developed for direct detection of *P. insidiosum* in tissue samples. The assay had high sensitivity (100%) and specificity (94%). Statistical analysis indicated that the IPS assay is very useful for ruling out pythiosis in suspected patients. The IPS assay requires routine immunoperoxidase staining reagents and laboratory equipments and can be performed easily by pathologists in rural hospitals where the disease is more prevalent.

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