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Protein A/G-based immunochromatographic test for serodiagnosis of pythiosis in human and animal subjects from Asia and Americas

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Abstract

Pythiosis is a life-threatening infectious disease of both humans and animals living in Asia, Americas, Africa, and parts of Australia and New Zealand. The etiologic pathogen is the fungus-like organism *Pythium insidiosum*. The disease has high mortality and morbidity rates. Use of antifungal drugs are ineffective against *P. insidiosum*, leaving radical surgery the main treatment option. Prompt treatment leads to better prognosis of affected individuals, and could be achieved by early and accurate diagnosis. Since pythiosis has been increasingly reported worldwide, there is a need for a rapid, user-friendly, and efficient test that facilitates the diagnosis of the disease. This study aims to develop an immunochromatographic test (ICT), using the bacterial protein A/G, to detect anti-*P. insidiosum* IgGs in humans and animals, and compare its diagnostic performance with the established ELISA. Eighty-five serum samples from 28 patients, 24 dogs, 12 horses, 12 rabbits, and 9 cattle with pythiosis, and 143 serum samples from 80 human

and 63 animal subjects in a healthy condition, with thalassemia, or with other fungal infections, were recruited for assay evaluation. Detection specificities of ELISA and ICT were 100.0%. While the detection sensitivity of ELISA was 98.8%, that of ICT was 90.6%. Most pythiosis sera, that were falsely read negative by ICT, were weakly positive by ELISA. In conclusion, a protein A/G-based ICT is a rapid, user-friendly, and efficient assay for serodiagnosis of pythiosis in humans and animals. Compared to ELISA, ICT has an equivalent detection specificity and a slightly lower detection sensitivity.

Key words: Pythiosis, *Pythium insidiosum*, Immunochromatography, Serodiagnosis.

Introduction

Pythiosis is a life-threatening infectious disease caused by the fungus-like, aquatic, oomycete organism *Pythium insidiosum*.[1–5] Most cases of pythiosis have been reported in humans, horses, dogs, cats, and cattle, but some other domestic and wild animals are also infected.[2,4,5] Tropical wetlands are the natural habitat of *P. insidiosum*, and the disease is likely to be acquired through the ingestion of contaminated water or direct contact with the pathogen to host surfaces.[6,7] Pythiosis has been found in Asian countries, that is, Thailand, Malaysia, and India.[4,8,9] However, the disease has been reported in the Americas (i.e., U.S.A., Costa Rica, Brazil),[10–12] Africa,[13] and parts of Australia and New Zealand.[14–16] Various forms of pythiosis have been observed, depending on the site at which the infection initiates, that is, artery, eye, skin, and gastrointestinal tract. Cutaneous/subcutaneous infection is the most common form of pythiosis in horses, whereas in dogs, gastrointestinal tract infection is more prevalent. In humans, *P. insidiosum* infections of arteries (vascular pythiosis) and eyes (ocular pythiosis) have been frequently reported.

Pythiosis has a high rate of mortality and morbidity.[2–5] Use of antifungal drugs are ineffective for the treatment of pythiosis. Extensive surgical removal of infected tissues is the main treatment option for cure. Prompt and effective treatment is required to promote better outcome for the affected individuals, and this could be achieved by early and accurate diagnosis of pythiosis. Several diagnostic methods are available for pythiosis.[2,4,5] Culture identification and PCR-based diagnostic assays are used for the direct detection of *P. insidiosum* in clinical samples.[8,17–22] However, these methods are time-consuming and require experienced personnel. In addition, culture identification often fails to isolate the organism from the infected tissue sample. Alternatively, serodiagnostic assays, including immunodiffusion (ID),[23,24] enzyme-linked immunosorbent assay (ELISA),[25–29] hemagglutination (HA),[30] and immunochromatographic test (ICT),[31] have been developed for detection of anti-*P. insidiosum* antibodies in serum samples. Among these assays, ELISA and ICT showed most favorable diagnostic performance for pythiosis.[28]

As alluded to earlier, pythiosis has been increasingly diagnosed in mammals, including humans and various domestic and wild animals worldwide.[2–5] There is a need for a serological test that could facilitate the diagnosis of *P. insidiosum* infections in these mammalian subjects. While ID is relatively slow and insensitive, HA has a poor diagnostic performance, and ELISA requires a multi-step procedure and expensive equipment, ICT appears to be a rapid, user-friendly, and efficient test format for serodiagnosis of pythiosis.[23–28,30,31] ICT has been developed to detect anti-*P. insidiosum* antibodies in serum samples particularly from human patients, and not that from other animals.[31] This limitation is, however, due to different reagents (i.e., host-specific anti-IgG antibodies) are needed to perform the test against sera obtained from different hosts. By using the bacterial protein A/G that binds various mammalian IgGs,[32,33] the present study aims to develop an ICT, using the bacterial protein A/G, to detect anti-*P. insidiosum* IgGs in humans and animals, and compare its performance with the established ELISA.[26,28,34]

Materials and methods

Serum samples

A total of 85 serum samples from 28 human patients (26 vascular, 1 ocular, and 1 cutaneous pythiosis), 24 dogs, 12 horses, 12 rabbits, and 9 cattle with pythiosis were recruited for ICT and ELISA analyses.[26,28,31,34] Diagnosis of pythiosis was based on: (i) culture identification of *P. insidiosum* from clinical specimens;[17] (ii) PCR-based assay or sequence homology analysis of *P. insidiosum* rDNA;[8,18,20] or (iii) detection of anti–*P. insidiosum* antibodies in serum samples by established serodiagnostic tests.[23–28,30,31,34] To serve as the control, a total of 143 serum samples were recruited from 80 human subjects (healthy blood donors [n = 62] and patients with thalassemia [n = 10], aspergillosis [n = 3], zygomycosis [n = 2], candidiasis [n = 1], cryptococcosis [n = 1], and histoplasmosis [n = 1]), 31 dogs (healthy dogs [n = 9], dogs with *Lagenidium giganteum* forma *caninum* infection

[n = 8], *Paralagenidium karlingii* infection [n = 6], zygomycosis [n = 3], aspergillosis [n = 1], blastomycosis [n = 1], cryptococcosis [n = 1], protothecosis [n = 1], and sporotrichosis [n = 1]), 12 healthy cattle, 10 healthy horses, and 10 healthy rabbits. All sera were kept frozen until use.

Antigen preparation

The *P. insidiosum* strain Pi-S, isolated from a Thai patient with pythiosis, was maintained on Sabouraud dextrose (SD) agar, and subcultured (at 37 °C) once a month until use. The antigen was prepared, using the protocol described by Krajaejun et al.[35] Briefly, several small pieces of SD agar containing growing *P. insidiosum* mycelium were cultured (at 37 °C for 10 days) with shaking (150 rpm) in a flask containing 200 ml of SD broth. The organism was killed by adding Thimerosal [final concentration: 0.02% (wt/vol)], and separated from cultured SD broth by filtration through a 0.22-μm pore size membrane (Durapore). To prepare the culture filtrate antigen (CFA), after adding protease inhibitors [PMSF (0.1 mg/ml) and EDTA (0.3 mg/ml)], the cell-free SD broth was concentrated ~80 fold using the Amicon 8400 apparatus and an Amicon Ultra-15 centrifugal filter (Millipore). Protein concentration of CFA was estimated by spectrophotometry. The CFA was stored at −20 °C until use.

Protein A/G-based immunochromatographic test

The protein A/G based ICT was produced at the Chulabhorn Research Institute and involved the following step:

(I) **Preparation of protein A/G colloidal gold conjugate:** Protein A/G (Prospec, Ness-Ziona, Israel) was coupled to a colloidal gold particle by pI-dependent passive adsorption. The 40-nm colloidal gold solution (Arista, Allentown, PA) was adjusted to pH 7.2 with 0.2 M Na_2CO_3 under gentle stirring. The gold suspension was divided into aliquots of 0.5 ml in 1.5 ml microcentrifuge tubes to which 30 μl of protein A/G (0.1 mg/ml) was added with gentle vortexing. The mixture was allowed to conjugate at room temperature for 30 min. The residual surface of the gold particle was blocked with 125 μl of 5% (wt/vol) casein dissolved in 5 mM sodium phosphate buffer (SPB) pH 7.4 for 15 min. The conjugation mixture was centrifuged at 6,000 × g at room temperature for 15 min, and the supernatant was discarded. The pellet was washed with 0.5% (wt/vol) casein and the suspended conjugated particle was centrifuged again under identical settings. After removing the supernatant, the pellet was re-suspended in a solution of 0.5% (wt/vol) casein containing 20% (wt/vol) sucrose in 5 mM SPB pH 7.4 to 2.5% of the original volume of colloidal gold suspension. The protein A/G colloidal gold conjugate (2.5 μl) was transferred to a piece of 2.5×4.0 mm glass fiber filter GF33; (Whatman Schleicher & Schuell, Dassel, Germany). The impregnated glass fiber was dried in a dehumidifier cabinet for 2 hours and was used to construct the ICT.

(II) **Immobilization of antigen and antibody onto a nitrocellulose membrane:** Immobilization of proteins on nitrocellulose membrane (NCmb) (AE99; Whatman Schleicher & Schuell, Dassel, Germany) was performed by passive physical adsorption in line pattern. A BioDot ZX1000^{TM} dispensing platform (BioDot, Irvine, CA) was used for this purpose. The transfer rate of the solution was set at 1 μl/cm. A 1.25×20 cm nitrocellulose membrane was lined along its length with 1 mg/mL CFA to form a test line and with 0.2 mg/ml normal rabbit IgG in 50 mM ammonium acetate buffer pH 4.5 to form a control line. The protein immobilized membrane was dried and blocked with 1% (wt/vol) BSA, 0.1% (wt/vol) trehalose in 10 mM SPB pH 7.4 and dried again in a dehumidifier cabinet.

(III) **Preparation of the sample pad:** The sample pad used in the ICT strip was made of paper (#903 Whatman Schleicher & Schuell, Dassel, Germany) previously immerged in 2% (wt/vol) Triton X-100, 0.05% (wt/vol) polyvinylpyrrolidone (PVP) in 50 mM Tris-HCl, pH 7.4 and dried in a dehumidifier cabinet.

(IV) **Composition and construction of the ICT strip:** The ICT strip system was assembled by utilizing five major components: the sample pad, the glass fiber impregnated with colloidal gold conjugate, the protein immobilized NC membrane, a wicking pad (3 MM chromatography paper, Whatman, Maidstone, England) and the plastic backing. The first 4 components were assembled with 1–2 mm overlap on the plastic backing support (G&L; San Jose, CA). The assembled card was then cut into 2.5 mm wide strips with a strip cutting machine (CM 4000 R; BioDot, Irvine, CA) (Figure 1).

(V) **Detection of anti-*P. insidiosum* antibodies in human and animal sera:** The ICT assay was carried out in a 96-well microtiter plate or a microtube. Human sera were diluted 1:10,000 (in 0.15 M PBS pH 7.4) and tested with ICT in Center #1 (Ramathibodi Hospital, Thailand), while animal sera were diluted 1:5,000 (in 0.15 M PBS pH 7.4) and tested with ICT in Center #2 (for samples from dogs; Louisiana State University, USA) and Center #3 (for samples from horses, dogs, cattle, and rabbits; Universidade Federal de Santa Maria, Brazil). The protein A/G-based ICT strip was dipped into a

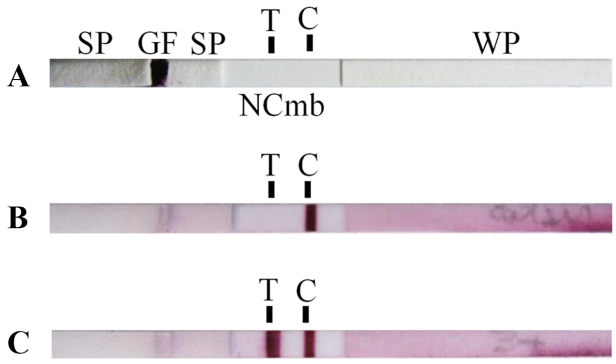

Figure 1. Schematic diagrams of the proteins A/G based immunochromatographic test (ICT) for detection of anti-*Pythium insidiosum* IgGs. Panel **A** (actual ICT strip) shows an untested ICT (either test or control line is not generated). Panel **B** depicts a negative result (only the control line is visible), whereas panel **C** exhibits a positive result (both test and control lines are visible). (Abbreviations: SP, sample pad; GF, glass fiber; WP, wicking pad; NCmb, nitrocellulose membrane; T, test line; and C, control line).

well containing 0.1 ml of each diluted serum sample in duplicate. The serum sample moved through the sample pad and the conjugate pad by capillary force. The mixture moved along the membrane immobilized with CFA acting as the test line. If anti-*P. insidiosum* antibody is present in the serum sample, the result is the formation of colored bands of colloidal gold conjugate at the test line and also the control line. On the other hand, a negative sample gives only one band at the control line. The developed signal of each ICT was read visually at 30 min. Detection sensitivity, detection specificity, and assay accuracy were calculated using the Microsoft EXCEL2013 program.

Enzyme-linked immunosorbent assay

ELISA for detection of anti-*P. insidiosum* antibodies in serum samples was carried out in three centers: (i) Department of Pathology, Faculty of Medicine, Ramathibodi Hospital, Mahidol University, Bangkok, Thailand (Center #1), using the ELISA protocol of Chareonsirisuthigul et al.[28] for testing human sera; (ii) Department of Veterinary Clinical Sciences, Louisiana State University, Baton Rouge, LA, USA (Center #2), using the ELISA protocol of Grooters et al.[26] for testing dog sera; and (iii) Departamento de Microbiologia e Parasitologia, Universidade Federal de Santa Maria, Santa Maria, RS, Brazil (Center #3), using the ELISA protocol of Santurio et al.[34] for testing sera from horses, dogs, cattle, and rabbits. The *P. insidiosum* antigen used for the ELISA performed in Center #1 was CFA (exoantigen),[28] while that used for the ELISA performed in Center #2 and Center #3 was soluble hyphal antigen.[26,34] The ELISA cut-off value was calculated based on the mean optical density (OD) of the control sera plus three SDs (Center #1 and #3), or the mean percent positivity of all control sample ODs (in relation to the strong positive control serum OD) plus three SDs (Center #2). Any samples with ELISA values above the cut-off were determined to be positive, while those below the cut-off were determined to be negative. Detection sensitivity, detection specificity, assay accuracy, and ELISA cut-off values were calculated using the Microsoft EXCEL2013 program.

Results

Development of a protein A/G-based ICT

An assembled protein A/G-based ICT is shown in Figure 1. CFA (crude protein extract from *P. insidiosum* strain Pi-S) and the commercially available normal rabbit IgGs were separately streaked as a straight line on a nitrocellulose membrane and served as "test" and "control" lines, respectively. Various IgG species in a human or animal serum samples were absorbed and migrated along the sample pad, and then the glass fiber, where complexes of IgGs and protein A/G conjugated with colloidal gold were formed. The IgG-protein A/G-colloidal gold complexes moved through the nitrocellulose membrane. The complexes containing anti-*P. insidiosum* IgGs captured the CFA blotted at the test line, and developed a purple signal of accumulated colloidal golds. The complexes lacking anti-*P. insidiosum* IgGs passed through the test line, without developing any signal. The normal rabbit IgGs, blotted at the control line, bound the protein A/G in the remaining complexes, and developed a purple signal.

After distribution of the ICT from Center #1 (Thailand) to Center #2 (USA) and Center #3 (Brazil), the assay can still function properly. After two years of storage at room temperature, the ICT can still effectively detect the anti-*P. insidiosum* IgGs in serum samples.

Performance comparison of ICT and ELISA

The established ELISA[26,28,34] and the protein A/G-based ICT had been independently performed to determine anti-*P. insidiosum* antibodies in sera from 108 humans, 55 dogs, 22 horses, 21 cattle, and 22 rabbits, with (n = 85) or without (n = 143; served as control) pythiosis, in the three centers: Center #1 (Department of Pathology, Ramathibodi Hospital, Mahidol University, Bangkok, Thailand), Center #2 (Department of Veterinary Clinical Sciences, Louisiana State University, Baton Rouge, LA, USA), and Center #3 (Departamento de Microbiologia e Parasitologia, Universidade Federal de Santa Maria, Santa Maria, RS, Brazil) (Table 1). The test results (i.e., ELISA value ranges, means, and cutoffs) and diagnostic performances (i.e., sensitivities,

Table 1. Diagnostic performances of ICT and ELISA tested against serum samples from humans (n = 108) or animals (n = 120), with and without pythiosis, in Center #1 (located in Thailand), Center #2 (USA), and Center #3 (Brazil).

Center	Host (Total sera)	Range of ELISA values (Mean)		ELISA cutoff value	ELISA			ICT		
		Pythiosis	Control		TP[a]/PC[b] (Sensitivity, %)	TN[c]/CC[d] (Specificity, %)	Accuracy (%)[e]	TP/PC (Sensitivity, %)	TN/CC (Specificity, %)	Accuracy (%)
1	Humans (108)	20.25–1.91 (13.47)	4.45–0.33 (1.55)	4.45	27/28 (96.4)	80/80 (100.0)	99.1	24/28 (85.7)	80/80 (100.0)	96.3
2	Dogs (44)	105.70–16.73 (62.38)	9.11–2.68 (5.01)	10.02	18/18 (100.0)	26/26 (100.0)	100.0	17/18 (94.4)	26/26 (100.0)	97.7
3	Dogs (11)	1.37–1.16 (1.25)	0.75–0.30 (0.47)	1.00	6/6 (100.0)	5/5 (100.0)	100.0	6/6 (100.0)	5/5 (100.0)	100.0
	Horses (22)	1.11–0.22 (0.80)	0.21–0.12 (0.17)	0.21	12/12 (100.0)	10/10 (100.0)	100.0	9/12 (75.0)	10/10 (100.0)	86.4
	Cattle (21)	0.37–0.17 (0.23)	0.16–0.09 (0.13)	0.16	9/9 (100.0)	12/12 (100.0)	100.0	9/9 (100.0)	12/12 (100.0)	100.0
	Rabbits (22)	0.33–0.19 (0.27)	0.15–0.09 (0.12)	0.18	12/12 (100.0)	10/10 (100.0)	100.0	12/12 (100.0)	10/10 (100.0)	100.0
1–3	Overall (228)	N/A[f]	N/A	N/A	84/85 (98.8)	143/143 (100.0)	99.6	77/85 (90.6)	143/143 (100.0)	96.5

[a]Number of cases with true positive (TP) results.
[b]Pythiosis cases.
[c]Number of cases with true negative (TN) results.
[d]Control cases.
[e]Accuracy (%), [(all cases with true positive and true negative results)/(all pythiosis and control cases)] × 100.
[f]N/A, not applicable.

specificities, and accuracies) of ELISA and the protein A/G-based ICT, according to host types (humans, dogs, horses, cattle, and rabbits), disease states (pythiosis or control), and assay-performing centers, are summarized in Table 1.

All control serum samples (n = 143) were tested negative by both the ELISA and protein A/G-based ICT. Among all 85 pythiosis sera, 77 samples were consistently tested positive by both serological assays. ELISA and ICT failed to detect anti-*P. insidiosum* antibodies in the same serum from a patient with ocular pythiosis (ELISA value: 1.91; ELISA cutoff: 4.45). Two patients with vascular pythiosis (ELISA values: 11.60 and 7.08), one patient with cutaneous pythiosis (ELISA value: 7.59), three horses with pythiosis (ELISA values: 0.26, 0.25, and 0.22; ELISA cutoff: 0.21), and a Center #2 dog with pythiosis (ELISA value: 16.73; ELISA cutoff: 10.02) were tested positive by ELISA but negative by ICT. One pythiosis serum from a human subject was weakly positive by ICT and ELISA (ELISA values: 6.28). Based on the results of all pythiosis and control sera, regardless of host types and assay-performing centers, ELISA showed 98.8% detection sensitivity, 100% detection specificity, and 99.6% accuracy, while ICT showed 90.6% detection sensitivity, 100% detection specificity, and 96.5% accuracy.

Discussion

The protein A/G-based ICT was successfully developed for the detection of specific anti-*P. insidiosum* IgGs in serum samples from humans and animals with pythiosis, which is a striking advantage over the previously-reported ICT that can detect only the IgGs from human subjects.[31] The diagnostic performance of ICT was compared to that of ELISA, a highly efficient assay established for the serodiagnosis of pythiosis in humans or animals.[26,28,34] Interpretation of ICT results depends on the presence or absence of the test line (which is subjectively read by the naked eye; Figure 1), while interpretation of ELISA results depends on an OD value above or below the cutoff (which was objectively quantitated by an ELISA plate reader). ICT and ELISA did not detect the anti-*P. insidiosum* IgG antibodies in the control sera from healthy individuals, as well as those from humans and animals with infections caused by other pathogens, including those share microscopic morphologies with *P. insidiosum* (i.e., *Lagenidium*, *Paralagenidium*, *Aspergillus*, and Zygomycetes) (Table 1). This finding indicates that both ELISA and ICT had no cross-reactivity with other pathogens and thus provides equivalently high detection specificity (100%).

Regardless of the sources of the sera tested, the overall detection sensitivity of ICT was considered high (~91%),

although slightly lower than that of ELISA (~99%; Table 1). This was due to some serum samples from several proven cases of human (n = 4), equine (n = 3), and canine (n = 1) pythiosis, being read negatively by ICT (i.e., no visible test line), but marginally positive by ELISA (i.e., ELISA values that were slightly above the cutoff; Table 1). The subjective nature of result interpretation could explain the limited detection sensitivity of ICT, especially when fewer anti-*P. insidiosum* antibodies were present in the serum sample, as indicated by a low ELISA value. The possibility of the presence of anti-protein A/G antibodies in the serum, that inhibits the formation of IgG-protein A/G-colloidal gold complexes, and leads to an absence of the test line, is unlikely. This can be explained by the fact that the ICT control line, generated by complex formation of the normal rabbit IgGs and the protein A/G conjugated with colloidal gold (Figure 1), was strongly developed in all serum samples tested, indicating that there was no anti-bacterial protein A/G antibodies in the samples. The reason for the false negative results was most likely due to the lower detection sensitivity of the ICT, as compared to that of ELISA. Poor host antibody responses can be observed in localized infections of the eye,[30,31] and could explain the failure of ICT and ELISA to detect anti-*P. insidiosum* antibodies in the serum from patient with ocular pythiosis. Therefore, the use of neither ICT nor ELISA is recommended for making a diagnosis of ocular pythiosis.

ELISA and ICT showed high accuracy (99.6% and 96.5%, respectively; Table 1), indicating that both assays reliably reported the true positive and true negative results. Here, we showed that ELISA is highly sensitive for the diagnosis of pythiosis, which was consistent with the reports of other investigators.[25–28,34] However, ELISA has a long turnaround time (>3 hr), and to perform this multi-step assay, it requires a specific secondary antibody (for each host type), experienced personnel, and special equipment. Such limitations of ELISA could be addressed by the successful development of the protein A/G-based ICT, which appears to be rapid (shorter turnaround time: ~30 min), highly-sensitive (91%), and easy-to-use assay that can facilitate serodiagnosis of pythoisis, especially in nonreference laboratories.

In conclusion, an ICT has been successfully developed for the serodiagnosis of humans and animals with pythiosis. ICT has a high detection sensitivity (91%), detection specificity (100%), and accuracy (97%). Yet, it was designed and manufactured to be a rapid, user-friendly, and efficient test. The current ICT has a long shelf storage life (at least two years), and it can be distributed worldwide, without effecting its performance. ICT could facilitate the diagnosis of pythiosis in most cases. However, if ICT is read negative in a suspected case of pythiosis, further analysis using a more sensitive assay (i.e., ELISA) is recommended.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

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