A PEPTIDE ELISA TO DETECT ANTIBODIES AGAINST *PYTHIUM INSIDIOSUM* BASED ON PREDICTED ANTIGENIC DETERMINANTS OF EXO-1,3-β-GLUCANASE

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Abstract. Human pythiosis is a life-threatening infectious disease caused by the oomycete *Pythium insidiosum*. Diagnosis of pythiosis relies on culture identification, serodiagnosis, and molecular-based assay. Preparation of a serodiagnostic test requires culture filtrate antigen (CFA) extracted from the live pathogen. A 74-kDa immunoreactive protein of *P. insidiosum*, is encoded by the exo-1,3-β-glucanase gene (*PinsEXO1*). PinsEXO1 protein is recognized by sera from pythiosis patients but not by sera from uninfected patients; therefore, this protein could be used to detect anti-*P. insidiosum* antibodies. In this study we aimed to: identify, synthesize, and evaluate an antigenic determinant (epitope) of PinsEXO1 to be used to serodiagnose pythiosis based on peptide ELISA, and to compare the diagnostic performance of that test with the current CFA-based ELISA. Two antigenic determinants of PinsEXO1 (Peptide-A and -B) were predicted using the PREDITOP program. The sera from 34 pythiosis patients and 92 control subjects were evaluated. Peptide-A, Peptide-B, and CFA-based ELISAs all had a specificity of 100%. Peptide-B ELISA had a sensitivity of 91% and an accuracy of 98% and both Peptide-A and CFA-based ELISAs had a sensitivity of 100% and an accuracy of 100%. Peptide-A is a more efficient epitope than Peptide-B, and can be used as an alternative antigen to develop a serodiagnostic assay for pythiosis.

Keywords: *Pythium insidiosum*, pythiosis, diagnosis, ELISA, exo-1,3-β-glucanase

INTRODUCTION

Pythium insidiosum is the only aquatic, fungus-like, oomycetous microorganism, belonging to the Stramenopiles of the supergroup Chromalveolates, that causes a devastating infectious disease, called pythiosis, in both humans and animals, including horses, dogs and cattle (Mendoza *et al*, 1996; Kamoun, 2003; Keeling *et al*, 2005). Morphologically, *P. insidiosum* looks like a filamentous fungus, but phylogenetically, it is different from true fungi (Kwon-Chung, 1994). *P. insidiosum* produces a special structure, called zoospore. The zoospore can swim to and attach itself to the host surface, geminate in the host tissue and cause pathology

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(Mendoza *et al*, 1993).

The first report of human pythiosis was in 1985 (Krajaejun *et al*, 2006b). Since then more cases have been reported, mostly from Thailand (Krajaejun *et al*, 2006b). Four different clinical features of human pythiosis have been described: 1) cutaneous pythiosis affecting the face or extremities as a granulomatous lesion or ulcer; 2) vascular pythiosis affecting arteries resulting in blood vessel occlusion and gangrene; 3) ocular pythiosis affecting eyes as a corneal ulcer; and 4) an unusual form of pythiosis affecting other internal organs, such as the gastrointestinal tract and brain (Krajaejun *et al*, 2006b). Thalassemic hemoglobinopathy is predisposing factor to pythiosis (Sathapatayavongs *et al*, 1989; Krajaejun *et al*, 2006b). The morbidity and mortality levels of pythiosis are high (Krajaejun *et al*, 2006b). Delayed diagnosis and lack of effective treatment are both problematic. Conventional antifungal drugs are ineffective against *P. insidiosum* infection (Krajaejun *et al*, 2006b). The treatment of choice is excision of the infected organ (Krajaejun *et al*, 2006b), which can cause other problems. Many patients died from advanced or uncontrolled infection. Early diagnosis can lead to prompt treatment and possibly a better prognosis.

Definitive laboratory diagnosis of pythiosis can be made by culture identification (Mendoza and Prendas, 1988; Chaiprasert *et al*, 1990), serodiagnosis (Krajaejun *et al*, 2002, 2009; Jindayok *et al*, 2009; Keeratijarut *et al*, 2009; Supabandhu *et al*, 2009; Chareonsirisuthigul *et al*, 2013), or molecular-based analysis (Badenoch *et al*, 2001; Grooters and Gee, 2002; Vanittanakom *et al*, 2004; Botton *et al*, 2011). Among the established diagnostic methods, serodiagnosis is more popular due to its convenience. Several serodiagnostic assays have been developed to diagnose pythiosis, including immunodiffusion (ID), enzyme-linked immunosorbent assay (ELISA), Western blot (WB), hemagglutination (HA), and immunochromatography (ICT) (Krajaejun *et al*, 2002, 2009; Jindayok *et al*, 2009; Supabandhu *et al*, 2009). Chareonsirisuthigul *et al* (2013) compared the above assays and found the ICT and ELISA methods had the best diagnostic sensitivity and specificity. HA and ID often give false negative results (Krajaejun *et al*, 2002, 2009; Jindayok *et al*, 2009; Chareonsirisuthigul *et al*, 2013). The ICT is a rapid and easy-to-use assay. However, the ICT and WB require reagents and equipment not routinely available in many clinical laboratories. Unlike some assays, ELISA results are unambiguously interpreted by an ELISA reader, which can prevent interpretation errors by laboratory personnel.

Preparation of serodiagnostic assays requires crude protein mixture, such as, culture filtrate antigen (CFA) or soluble antigen from broken hyphae (SABH) extracted directly from the live pathogen. Batch-to-batch variation and significant degradation of the crude protein extract may lead to lower sensitivity and specificity of the assay. An alternative to the crude protein extract, synthetic peptides, containing a specific antigenic determinant (epitope), have been useful for developing an immunoassay to detect antibodies against a variety of pathogens (Gümara and Haro, 2007; Velumani *et al*, 2011). Additionally, peptides are not infectious and can be commercially synthesized at a high level of purity and in unlimited amounts.

We identified a specific 74-kDa immunoreactive protein in the crude extract of *P. insidiosum* by Western blot (Krajaejun *et al*, 2006a). By using proteomic and genetic approaches, we later found this

protein is encoded by a putative exo-1,3-β-glucanase gene (*PinsEXO1*) (Krajaejun *et al*, 2010). PinsEXO1 protein is recognized by the sera of patients with pythiosis, but not by the sera of uninfected patients (Krajaejun *et al*, 2006a, 2010). Thus, PinsEXO1 could be a novel antigenic target used for specific detection of anti-*P. insidiosum* antibodies. Therefore, in the current study, we determined to: identify, synthesize, and evaluate an antigenic determinant of PinsEXO1 to serodiagnose pythiosis based on a peptide ELISA, and to compare the diagnostic performance of this new peptide-based ELISA with the established CFA-based ELISA (Chareonsirisuthigul *et al*, 2013).

MATERIALS AND METHODS

Serum samples

Thirty-four serum samples were obtained from vascular (*n*=33) and cutaneous (*n*=1) pythiosis patients, diagnosed based on successful isolation of *P. insidiosum* from infected tissue (Chaiprasert *et al*, 1990), or detection of anti-*P. insidiosum* antibodies in their sera (Pracharktam *et al*, 1991; Krajaejun *et al*, 2002, 2009; Jindayok *et al*, 2009). Control samples (*n*=92) were also obtained; they were comprised of: 1) serum samples from healthy blood donors at the Blood Bank Division, Ramathibodi Hospital (*n*=56); 2) serum samples from thalassemic patients without pythiosis (*n*=6); 3) serum samples from patients with positive antinuclear antibodies (*n*=9) and rheumatoid factor (*n*=2); and 4) serum samples from patients positive for other infectious diseases (2 with zygomycosis, 1 with aspergillosis, 1 with candidiasis, 1 with histoplasmosis, 1 with cryptococcosis, 2 with syphilis, 2 with human immunodeficiency virus infection, 6 with hepatitis B virus infection, and 3 with hepatitis C virus infection). Positive and negative control serum samples were also included in the evaluation. All serum samples were kept at -20ºC until used.

Preparation of culture filtrate antigen

Culture filtrate antigen (CFA) was prepared from the *P. insidiosum* strain Pi-S (isolated from a Thai patient with vascular pythiosis), using the method described previously (Jindayok *et al*, 2009; Krajaejun *et al*, 2009). Ten small pieces of agar with actively growing mycelia (3-days culture on Sabouraud dextrose agar) were transferred to a 500-ml flask containing 100 ml of Sabouraud dextrose broth, and shaken (150 rpm) at 37ºC for 10 days. Merthiolate was added to the culture (final concentration, 0.02% wt/vol). The culture was filtered through a Durapore filter membrane (pore size, 0.22-μm). PMSF (0.1 mg/ml) and EDTA (0.3 mg/ml) were added to the filtered broth. The resulting CFA was concentrated ~80-fold using an Amicon 8400 apparatus with an Amicon Ultra-15 centrifugal filter (Millipore, Bedford, MA). The protein concentration was measured using a spectrophotometer. CFA was kept at -20ºC until use.

Epitope prediction and biotin-labelled peptides

A partial sequence of the putative exo-1,3-β-glucanase encoding gene (*PinsEXO1*) for *P. insidiosum* (accession number: GU994093.1) (Krajaejun *et al*, 2010) was BLAST searched using a local transcriptome database for this pathogen (unpublished data). The resulting longer gene sequence was subject to protein translation using the ESTscan program (Iseli *et al*, 1999), and then epitope prediction using the PREDITOP program (the program is based on turn predictions and the peptide hydrophilic) (Pellequer and Westhof, 1993). Peptides with predicted

antigenic determinant were synthesized (>95% purity) and biotin-labelled by PEPNOME limited (Shuzhou City, China).

CFA-based ELISA

The CFA-based ELISA was performed using the method of Chareonsirisuthigul *et al* (2013), with some modifications. Briefly, a 96-well polystyrene plate (Costar, Bloomington, MN) was coated with 100 µl of CFA (5 µg/ml) in 0.1 M carbonate buffer (pH 9.6) and incubated at 4ºC overnight and then washed four times with phosphate buffer solution (pH 7.4) containing 0.5% Tween20 (PBS-T). The CFA-coated plate was blocked with 100 µl of casein buffer [1% casein in phosphate buffer solution (pH 9.6)] at 37ºC for 1 hour, and washed four times with PBS-T. A test serum sample diluted 1:800 in the casein buffer was added to each well (100 µl/well), incubated at 37ºC for 2 hours and then washed four times with PBS-T. The positive and negative control sera were tested in parallel. Each serum sample was tested in duplicate. Horseradish peroxidase-conjugated goat anti-human immunoglobulin G (Jackson Immuno Research, West Grove, PA) diluted 1:100,000 in the casein buffer was added to each well (100 µl/well), incubated at 37ºC for 1 hour and then washed four times with PBS-T. Freshly-prepared chromogen [20 µl of 0.6% TMB and 1 ml of 0.009% hydrogen peroxide in acetate buffer solution (25 µmol/ml)] was added to each well (100 µl/well) and incubated at room temperature for ~5 minutes. The enzymatic reaction was stopped with 100 µl of 0.3 N sulfuric acid.

Peptide-based ELISA

A 96-well NeutrAvidin coated plate (Pierce, Rockford, IL) was coated with 100 µl of the biotin-labelled peptide-A or peptide-B (5 µg/ml) in carbonate buffer and incubated at 4ºC overnight. The coated plate was then washed four times with PBS-T. A test serum sample diluted 1:1,600 in the casein buffer was added to each well (100 µl/well), incubated at 37ºC for 2 hours and then washed four times with PBS-T. The positive and negative control sera were tested in parallel. Each serum sample was tested in duplicate. Horseradish peroxidase-conjugated goat anti-human immunoglobulin G (Jackson Immuno Research, West Grove, PA) diluted 1:2,000 in the casein buffer was added to each well (100 µl/well), incubated at 37ºC for 1 hour and then washed four times with PBS-T. The chromogen solution (prepared as above) was added to each well (100 µl/well) and incubated at room temperature for ~5 minutes. The enzymatic reaction was stopped with 100 µl of 0.3 N sulfuric acid.

Measurement of ELISA signal and statistical analysis

The optical density (OD) of each sample was measured using an Infinite 200 Pro ELISA reader (Tecan, Groedig, Austria) at a wavelength of 595 nm. The mean OD value for each sample was corrected for the OD of the buffer control (the casein buffer). The OD value of each serum sample was divided by the OD value of the same negative control serum, to obtain an ELISA value (EV). Sensitivity (true positive (TP) / (false negative (FN) + TP) x 100), specificity (true negative (TN) / (false positive (FP) + TN) x 100), accuracy [(TP + TN) / (TP + TN + FP + FN) x 100], mean value for the EV and standard deviation (SD) were calculated using the Microsoft program Excel 2013.

RESULTS

Antigenic determinant prediction of *P. insidiosum* glucanase

To obtain a longer sequence, the *Pin-*

MALAVAVMALSGSVSANKQQRFLIRAHSNGSSSHAATGAGSTPAAEPEAKAEPKPKQK
KEVVRDAKWYWEHEEDPRFAAATGSFKAFTPDDATQTCSHDTHATPFNKQVRGANLGG
WLVLEPWITPSLFYQFLGTQERFGDSAPNKTAMDSYTFCTALGKEEANRQLRVHWANW
VTEDDIAEMAAAGVNSLRVPVGDWMFTPYEPYIGCTDGAIEELDRVADLAHKYGMDIL
IDIHGLIGSQNGFDNSGKTSAVXVDVDREHAARGHDDVRALAIRQAEWAGTFDPAKHA
YTSIN**YGNLNQSLTAVEEIVKRYASHPAVLGLQPVNEPWELTPIKVLKTYYWKSYKRV**
KALAPHWKFVLHDSFRFGREFWLDFMRGCPDIAIDTHIYQAWMNPGTKEDFYSNACQQ
KYTITDIENAVMPVIVGEWSLGTDNCAMWLNGFNDNLPGFPKVICQLRHCPVESTYLG
KGFPGTPLDTTKPIQGPYGTGTSGPSFGLCPVNSNLTFGQKTPEDELKFMKNLMSKKL
NAWLLGHGFYFWNFKTELDTRWDFLALVRAGVMPKNISDYDDADGIFDACEREDKGDF
VCRAKRGVKPFELENGLAYACNAEGVDCSNVKQKYLTLLEQCDYAFNEYWHQYREKGA
TCDFGGAAHLLSVPSNTTATGVKPSQINTKSAGIDSADASGASGWWPPTTAALIAYIV
GGVAVLAAVGGAIFQWRQRRLRRQQYNPIGXPIGTNSARLTAVCYAPLLAATHSS

Fig 1–The UN05080-translated, 751-amino acid-long protein sequence of the putative exo-1,3-β-glucanase of *P. insidiosum*, PinsEXO1. Bold letters are 307 amino acids of the partial PinsEXO1 protein [accession number, ADI86643.1 (Krajaejun *et al*, 2010)] used to BLAST search the local *P. insidiosum* transcriptome database (unpublished data) to obtain an extended PinsEXO1 protein sequence. Gray boxes indicate two antigenic determinants (Peptide-A and Peptide-B, respectively) of PinsEXO1, predicted by the PREDITOP program (Pellequer and Westhof, 1993).

sEXO1 partial sequence (accession number, GU994093.1) (Krajaejun *et al*, 2010) was BLAST searched against our local 454-generated *P. insidiosum* transcriptome database, comprising 26,735 unigenes (unpublished data). The BLAST analysis best matched (E-value, 0.0; identity, 99%; query coverage, 100%) the transcript number UN05080 (submitted to the DNA Data Bank of Japan under the accession number FX532070). The corresponding partial *P. insidiosum* glucanase protein (PinsEXO1; 307 amino acids long; accession number, ADI86643.1) identically matched the UN05080-deduced 751-amino acid-long protein (E-value, 0.0; sequence identity, 100%; query coverage, 100%) (Fig 1). The program PREDITOP (Pellequer and Westhof, 1993) predicted two antigenic determinants in the UN05080 protein sequence: Peptide-A (GLIGSQNGFDNSGKT) and Peptide-B (GPYGTGTSGPSFGL) (Fig 1). Peptide-A and Peptide-B were synthesized (> 95% purity) and biotin-labelled (at the N-terminus).

Diagnostic performance of CFA- and peptide-based ELISAs

To ensure accurate results, all the ELISA analyses included the same positive and negative control sera. The mean EVs for the pythiosis serum samples tested (n=34) by the CFA-based, Peptide-A, and Peptide-B ELISAs were: 145.9 (range, 16.1-236.9; SD, 57.6), 7.7 (range, 2.8-11.7; SD, 2.5), and 2.3 (range, 0.7-5.4; SD, 1.0), respectively. The mean EVs for the control serum samples from healthy blood donors (n=56), thalassemic patients without pythiosis (n=6), patients with autoimmune diseases (n=11), and patients with other infectious diseases (n=19) by the CFA, Peptide-A and Peptide-B ELISAs were: 2.2 (range, 0.0-10.8; SD, 2.1), 0.9 (range, 0.2-2.3; SD, 0.6), and 0.4 (range, 0.1-1.2; SD, 0.3), respectively. Five cutoff points were calculated based on the summation of the mean EV for the control sera at 2, 3, 4, 5 and 6 SDs. Each cutoff point gave different sensitivity and specificity results (Table 1). An optimal cutoff EV was selected based

Table 1
Diagnostic performance of CFA- and peptide-based ELISA for detection of anti-*P. insidiosum* antibodies in serum samples from 34 human pythiosis and 92 control cases.

Cutoff value[a]	CFA-based ELISA (Mean EV, 2.2; SD, 2.1)[a]			Peptide-A ELISA (Mean EV, 0.9; SD, 0.6)[a]			Peptide-B ELISA (Mean EV, 0.4; SD, 0.3)[a]		
	Sensitivity[b] (%)	Specificity[c] (%)	Accuracy[d] (%)	Sensitivity[b] (%)	Specificity[c] (%)	Accuracy[d] (%)	Sensitivity[b] (%)	Specificity[c] (%)	Accuracy[d] (%)
Mean + 2 SDs	100.0	95.6	96.8	100.0	93.4	95.2	93.9	93.4	93.5
Mean + 3 SDs	100.0	97.8	98.4	**100.0**	**100.0**	**100.0**	**90.9**	**100.0**	**97.6**
Mean + 4 SDs	100.0	98.9	99.2	93.9	100.0	98.4	87.9	100.0	96.8
Mean + 5 SDs	**100.0**	**100.0**	**100.0**	90.9	100.0	97.6	63.6	100.0	90.3
Mean + 6 SDs	100.0	100.0	100.0	90.9	100.0	97.6	51.0	100.0	87.9

CFA, culture filtrate antigen; ELISA, enzyme-linked immunosorbent assay; EV, ELISA value; SD, standard deviation.
[a] Mean ELISA value (EV) and standard deviation (SD) of the control sera (n=92)
[b] Sensitivity = true positive/(false negative+true positive) x 100
[c] Specificity = true negative/(false positive+true negative) x 100
[d] Accuracy (ability of an assay to provide true positive and true negative results) = (true positive+true negative)/(true positive+true negative+false positive+false negative) x 100.

on the highest accuracy obtained (Table 1). The summation of mean and 5 SDs (EV = 12.7) was the optimal cutoff value for CFA-based ELISA (accuracy = 100%). The mean plus 3 SDs was selected as the optimal cutoff value for both Peptide-A (EV cutoff = 2.7; assay accuracy = 100%) and Peptide-B (EV cutoff = 1.3; assay accuracy = 98%) ELISAs. With these cutoff values, detection specificities for the CFA, Peptide-A, and Peptide-B ELISAs were all 100%. The detection sensitivities for the CFA and Peptide-A ELISAs were 100% and the Peptide-B ELISA was 91%.

DISCUSSION

PREDITOP is a useful program for identifying peptides with potential epitopes in a protein of interest (Pellequer and Westhof, 1993). The epitope is capable of inducing antibody production, and the antibody produced can bind to the corresponding epitope. The PREDITOP program is based on "turn prediction"; the peptide chosen is invariably hydrophilic (Pellequer and Westhof, 1993). Thus, the peptide is likely to appear on the protein surface, and could lead to robust antibody production or binding. Two antigenic epitopes (Peptide-A and Peptide-B) were predicted in the UN05080-translated protein (Fig 1). Peptide-A and Peptide-B were tested for their ability to detect pythiosis by determining anti-*P. insidiosum* antibodies in sera of patients with pythiosis. ELISA was selected as the assay platform because of its superior diagnostic performance over the other assays (Chareonsirisuthigul *et al*, 2013). Additionally, while the result of other assays is subjectively determined by visual assessment, ELISA results are unambiguously determined by a machine, which can prevent some interpretation error by laboratory personnel (Chareonsirisuthigul *et al*, 2013).

Peptides, being a short stretch of amino acids, can be poorly coated onto an ELISA plate, or may adhere to the plastic surface in a way that masks its antigenic epitope. This can lead to limited interaction of the peptide and its corresponding antibody. To prevent this, Peptide-A and Peptide-B were conjugated to biotin. Biotin is efficiently and specifically captured by avidin, a molecule pre-linked on the ELISA plate. This strategy may enhance an efficacy of ELISA plate coating, and better promote peptide-antibody interaction. The Peptide-A and Peptide-B ELISAs were performed on 34 pythiosis patients and 92 control patients. Both assays could discriminate between the pythiosis and control samples. However, the overall diagnostic performance of the Peptide-A ELISA (100% sensitivity, 100% specificity and 100% accuracy) was better than the Peptide-B ELISA (91% sensitivity, 100% specificity and 98% accuracy). Although the epitope prediction program, PREDITOP, can increase the chance of successfully detecting a peptide candidate with an antigenic epitope, it cannot guarantee all the predicted epitopes (Peptide-A and Peptide-B) are efficient in antibody production and binding. Thus, evaluation of more than one peptide is necessary. Peptide-A was a more efficient epitope than Peptide-B. We collected as many pythiosis serum samples as possible for this study, but the disease is rare and the number of samples was limited (n=34). Further evaluation using more pythiosis serum samples would be useful to more accurately assess the detection sensitivities of the assays.

The recently developed CFA-based ELISA is becoming a reference assay for serodiagnosis of pythiosis (Chareonsirisuthigul *et al*, 2013). The diagnostic performances of the Peptide-A ELISA and

the CFA-based ELISA were compared. The mean EV of the pythiosis serum samples determined with the CFA-based ELISA (EV = 145.9) was 19-fold higher than the Peptide-A ELISA (EV = 7.7). The EV detected with the CFA-based ELISA and the Peptide-A ELISA in the control sera were 2.4 fold different from each other. This may be due to the presence of hundreds of different antigenic epitopes in the CFA protein ELISA, compared to only one epitope in the Peptide-A ELISA. Both assays provided equivalent diagnostic performances (100% sensitivity, 100% specificity and 100% accuracy) (Table 1).

In conclusion, we report here the use of synthetic PinsEXO1 peptides to serodiagnose human pythiosis. The detection specificity of the CFA-based, Peptide-A, and Peptide-B ELISAs were equally high (100%). The Peptide-B ELISA had lower sensitivity (91%) and accuracy (98%), than the Peptide-A and CFA-based ELISAs (both methods 100% detection sensitivity and 100% accuracy). Thus, synthetic Peptide-A can be used as an alternative antigen for development of a serodiagnostic test for pythiosis. Synthetic peptides can address the concerns of batch-to-batch variations and degradation of the crude protein extract. Since peptides are non-infectious and commercially-available, they can be safely handled and used in the diagnostic laboratory.

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