AMERICAN SOCIETY FOR MICROBIOLOGY | Journal of Clinical Microbiology®

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Monitoring Anti-*Pythium insidiosum* IgG Antibodies and (1→3)-β-D-Glucan in Vascular Pythiosis

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ABSTRACT Despite aggressive treatment, vascular pythiosis has a mortality rate of 40%. This is due to delays in diagnosis and a lack of effective monitoring tools. To overcome this drawback, serum beta-D-glucan (BG) and *P. insidiosum*-specific antibody (*Pi*-Ab) were examined as potential monitoring markers in vascular pythiosis. A prospective cohort study of vascular pythiosis patients was carried out from January 2010 to July 2016. Clinical information and blood samples were collected and evaluated by the BG and *Pi*-Ab assays. Linear mixed-effect models were used to compare BG and *Pi*-Ab levels. The *in vitro* susceptibility test was performed with all *P. insidiosum* isolates from culture-positive cases. A total of 50 patients were enrolled: 45 survived and 5 died during follow-up. The survivors had a significantly shorter time to medical care ($P < 0.0001$) and a significantly shorter waiting time to the first surgery ($P < 0.0001$). There were no differences in BG levels among the groups at diagnosis ($P = 0.33$); however, BG levels among survivors were significantly lower than those of the deceased group at 0.5 months ($P < 0.0001$) and became undetectable after 3 months. Survivors were able to maintain an enzyme-linked immunosorbent assay (ELISA) value (EV) of *Pi*-Ab above 8, whereas the EV among deceased patients was less than 4. *In vitro* susceptibility results revealed no synergistic effects between itraconazole and terbinafine. This study showed that BG and *Pi*-Ab are potentially valuable markers to monitor the disease after treatment initiation. An unchanged BG level at 2 weeks after surgery should prompt an evaluation for residual disease.

KEYWORDS pythiosis, biomarkers, (1→3)-β-D-glucan, *P. insidiosum*-specific antibody, therapeutic monitoring

Received 11 April 2018 **Returned for modification** 4 May 2018 **Accepted** 22 May 2018

Accepted manuscript posted online 30 May 2018

Citation Worasilchai N, Permpalung N, Chongsathidkiet P, Leelahavanichkul A, Mendoza AL, Palaga T, Reantragoon R, Finkelman M, Sutcharitchan P, Chindamporn A. 2018. Monitoring anti-*Pythium insidiosum* IgG antibodies and (1→3)-β-D-glucan in vascular pythiosis. J Clin Microbiol 56:e00610-18. https://doi.org/10.1128/JCM.00610-18.

Editor David W. Warnock

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Pythium insidiosum, a fungus-like organism, is an aquatic oomycete in the kingdom Stramenopila. *P. insidiosum* lives in moist soil and stagnant water. During its infectious stage, *P. insidiosum* produces zoospores which can invade humans and other animals, e.g., horses, dogs, and birds. The first case of human subcutaneous pythiosis was reported in Thailand in 1985, and Thailand has the highest incidence of this disease (1). Currently, the increased trend of human pythiosis cases has been revealed not only in Thailand but also in other countries in Asia (2–6), Australia (7–9), North America (10), and South America (11). Vascular pythiosis is the most common manifestation of human pythiosis, which almost always occurs in patients with underlying hemoglobinopathy complicated by hemochromatosis (1, 12–14). Despite state-of-the-art treatments, the mortality rate of vascular pythiosis is still 40% (12), and its morbidity, among survivors, is severe due to the need for aggressive amputation to remove all infected tissues (13).

Currently, the following diagnostic criteria are used: presence of typical pathological features, successful isolation of *P. insidiosum* (13), positivity for *P. insidiosum*-specific antibody (*Pi*-Ab) by Western blotting (15) or enzyme-linked immunosorbent assay (ELISA) (16), and positive PCR using internal transcribed spacer and cytochrome oxidase II (PCR-ITS/*COX*2) primers (17–19). Positive culture in conjunction with zoospore production remains the gold standard (20).

Treatment practices vary across different institutions (13, 16). At King Chulalongkorn Memorial Hospital (KCMH), aggressive surgery and systemic antifungal therapy with a combination of itraconazole and terbinafine (ITC-TRB), as well as the adjunctive use of *P. insidiosum* antigen (PIA) immunotherapy (PIAI), have been recommended in all vascular pythiosis patients under our institutional research protocols (13). PIA, a crude protein antigen prepared from *P. insidiosum*, has been used in humans as immunotherapy since 1998; PIAI was successfully used to treat patients with carotid artery disease for which surgical intervention was not possible (21). A year course of ITC-TRB has been recommended based on a case report of *P. insidiosum* periorbital cellulitis in a child who was completely cured by ITC-TRB (22). Our previous study, however, revealed no synergistic effect of ITC-TRB for *P. insidiosum* isolates in Thailand (13). In clinical practice, susceptibility testing for this pathogen was not routinely performed, and antifungal treatment was not affected by the MICs, given no standardized interpretation.

During the 1-year treatment course, patient history and physical examination at each clinic visit are the main follow-up tools. Culture or detection of *P. insidiosum* DNA at each clinic visit is very unlikely to be successful without invasive surgery to obtain infected tissues. Without standard serologic or inflammatory markers, the sensitivity for detection of early signs of treatment failure or residual disease is low. This study was conducted as a preliminary characterization to examine the potential of serum β-D-glucan (BG) and *P. insidiosum*-specific antibody (*Pi*-Ab) as monitoring markers in vascular pythiosis. *In vitro* susceptibility testing against amphotericin B, voriconazole, itraconazole, fluconazole, anidulafungin, caspofungin, and terbinafine was also performed with all *P. insidiosum* isolates from culture-positive cases.

(This study was partially presented at the 8th Trends in Medical Mycology [TIMM] in Belgrade, Serbia [23].)

MATERIALS AND METHODS

Study design. We performed a prospective cohort study of vascular pythiosis patients who had received a combination therapy of surgery, systemic antifungal agents, and immunotherapy with PIAI according to the KCMH research treatment protocol from January 2010 to July 2016.

This study was approved by the Chulalongkorn University Institutional Review Board based on international guidelines for human research protection, such as the Declaration of Helsinki, the Belmont Report, the Council for International Organizations of Medical Sciences (CIOMS) Guideline, and the International Conference on Harmonization in Good Clinical Practice (ICH-GCP).

Patients. All proven vascular pythiosis patients over the age of 18 years who received treatment with surgery, systemic itraconazole (Sporal) at 100 mg three times daily, systemic terbinafine (Lamisil) at 250 mg twice daily, and immunotherapy with PIAI for a 1-year course according to the KCMH protocols were enrolled in this study. The diagnosis of vascular pythiosis was confirmed by one of the following

TABLE 1 Characteristics of vascular pythiosis patients

Parameter	Value for group[a]		*P* value
	Survived ($n = 45$)	Deceased ($n = 5$)	
Patient-related parameters			
Age (yr)	33.6 ± 10.7	49.2 ± 17.2	0.006
Male sex	25 (56)	4 (80)	0.29
Occupation			0.39
Agriculture related	39 (86.7)	5 (100)	
Non-agriculture related	6 (13.3)		
History of water exposure within 3 mo	41 (91.1)	5 (100)	0.49
Underlying disease			0.87
α-Thalassemia	3 (6.7)		
β-Thalassemia	3 (6.7)		
β-Thalassemia hemoglobin E disease	32 (71.1)	4 (80)	
Hemoglobin H-constant spring	2 (4.4)		
Hemoglobin H disease	5 (11.1)	1 (20)	
Serum ferritin (ng/ml)	1,388.40 ± 653	1,676 ± 402.4	0.34
Period from onset of disease to first medical attention (mo)	1.9 ± 0.7	4 ± 0.7	<0.0001
Period from diagnosis to first definitive surgery (mo)	0.6 ± 0.2	1.5 ± 1.2	<0.0001
Disease and treatment-related parameters			
Location of lesions			0.05
Brachial artery	1 (2.2)		
Radial artery	11 (24.4)		
Ulnar artery	1 (2.2)		
Femoral artery	17 (37.8)	4 (80)	
Anterior tibial artery	9 (20)		
Posterior tibial artery	3 (6.7)		
Iliac artery	3 (6.7)		
External carotid artery		1 (20)	
Surgical procedures			<0.0001
Amputation	45 (100)	2 (40)	
Debridement		3 (60)	
Antifungal agents			0.17
Itraconazole alone	5 (11.1)	2 (40)	
Itraconazole + terbinafine	34 (75.6)	3 (60)	
SSKI[b] + terbinafine	6 (13.3)		
Duration of antifungal treatment (mo)	5.9 ± 4.6	1.4 ± 0.9	0.04
Iron chelation drug	45 (100)	5 (100)	
Clinical signs/symptoms post-treatment initiation			
Fever > 38.2°C		3 (60)	0.008
Arterial insufficiency syndrome (claudication, paresthesia, gangrenous ulceration)		2 (40)	0.008
Mass at surgical sites (arterial aneurysm)		1 (20)	0.1
New skin lesions			
Inflammation/infection at surgical sites		3 (60)	0.008

[a]Unless otherwise indicated, the values are number (%) of patients.
[b]SSKI, saturated solution of potassium iodide.

accepted diagnostic criteria: (i) successful isolation of *P. insidiosum*, (ii) positive results for PCR-ITS/*COX*2 either from the isolates or directly from the clinical specimens (17–19). Patients with positive cultures were classified as proven vascular pythiosis cases, and patients with only positive PCR were classified as probable vascular pythiosis cases. Informed consent was obtained from all patients, and the detailed information regarding the current treatment protocol including PIAI was provided as an investigational treatment under the research protocol.

Serum and clinical data collection. Eligible patients were enrolled in the study at the time of diagnosis. The following data were collected at the enrollment visit: patient characteristic and vascular pythiosis-related data (anatomical lesions, disease duration, type of surgery, antifungal treatment, and iron-chelating therapy). During the follow-up course, signs and symptoms of possible residual disease, including fever, pain, skin rash, mass at surgical sites, arterial insufficiency syndrome, and inflammation at the surgical site, were recorded (Table 1).

Patients were followed up for 1 year after the primary dose of PIAI, and their follow-up visits were synchronized with their PIAI schedule as described below. If patients were transferred back to their home provinces, PIAI was sent to their local hospitals and the case record forms were filled out by their primary care providers. At each follow-up visit, patient blood samples were collected by using acid citrate dextrose (ACD) vacuum tubes prior to PIAI administration and the samples were delivered back to KCMH

within 24 h. Patient sera were prepared from their blood samples by centrifugation at 1,500 × *g* for 15 min and kept at −80°C. Age- and sex-matched sera obtained from thalassemia patient donors without active bacterial or fungal infections and healthy donors were used as a negative control for both BG and *Pi*-Ab testing. To prove the specificity of the *Pi*-Ab assay based on the in-house ELISA established by KCMH (KCMH-ELISA), a total of 30 nonpythiosis patient serum samples, i.e., from patients with nocardiosis or other fungal infections (candidiasis, cryptococcosis, aspergillosis, talaromycosis, or histoplasmosis), proven by nucleic acid sequence analysis, were tested in parallel.

***P. insidiosum* immunotherapy preparation and schedule.** PIAI was prepared according to the method described by Mendoza et al. (24). To avoid the effect of batch-to-batch variation, all PIA used in this study was from the same batch. One milliliter of 2 mg/ml PIA was administered via the subcutaneous route according to the following PIAI schedule: the first dose (time zero) was administered as soon as the definitive diagnosis was established, and subsequently, six booster doses were administered at 0.5, 1, 1.5, 3, 6, and 12 months.

Beta-D-glucan assay. Serum BG was quantitated, in duplicate, by using the Fungitell assay (Associates of Cape Cod, Inc., Falmouth, MA, USA) according to the manufacturer's instructions. Pooled serum samples from 40 thalassemia patient donors and 40 healthy donors were run in parallel as a negative control. The range of the Fungitell assay was 31 pg/ml to 500 pg/ml. Samples with BG levels out of the indicated range were reported and processed in the statistical analyses as 31 pg/ml and 500 pg/ml for levels of <31 pg/ml and >500 pg/ml, respectively. Additional dilutions were not performed. Serum BG levels of <60 pg/ml, 60 to 79 pg/ml, and ≥80 pg/ml were interpreted as negative, indeterminate, and positive, respectively (25, 26).

***P. insidiosum*-specific antibody assay.** Serum *Pi*-Ab levels were monitored by the KCMH-ELISA, a semiquantitative assay, in quadruplicate. Microtiter plates (Polysorp-Nunc, NY) were coated with 0.1 ml of 2 mg/ml PIA in bicarbonate buffer overnight at 4°C and then washed with 1% (vol/vol) Tween 20 in phosphate-buffered saline (1% PBS-T; PBS consists of 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 2 mM KH_2PO_4) and blocked with 2% skim milk in PBS-T for 1 h at 37°C. After washing, 100 μl of serum in 0.5% skim milk was added to each well and incubated for 1 h at 37°C. Horseradish peroxidase-conjugated rabbit anti-human immunoglobulin G (Dako, Denmark) in PBS-T and *O*-phenylenediamine dihydrochloride in citric acid with 30% H_2O_2 were used as a secondary antibody and a substrate, respectively. Reactions were quenched by 200 μl of 1 N H_2SO_4. The optical density at 490 nm (OD_{490}) was measured. Pooled serum samples from 40 thalassemia patient donors and 40 healthy donors were analyzed as a negative control (27).

Optimal serum dilution for *Pi*-Ab assay. The optimal serum titer was defined as the titer that presented the best discrimination of OD_{490} values among pythiosis patient sera at each PIAI injection time point. Pooled serum samples from five vascular cases, randomly selected from each region in Thailand, were tested. Twofold serial dilutions (1:50 to 1:25,600) of those pooled sera were tested by ELISA in quadruplicate. The correlation between the OD_{490} value and the serum dilution was plotted using the GraphPad Prism 5 program (GraphPad Software, Inc., La Jolla, CA, USA).

***Pi*-Ab assay result analysis.** To standardize the ELISA result for each testing batch, the OD_{490} values were normalized to the ELISA value (EV) according to the following formula: $EV = (OD_{sample} - OD_{background})/(OD_{control} - OD_{background})$.

Statistical analyses. Statistical analyses were conducted by SAS version 9.4 (SAS Institute, Cary, NC, USA). The *t* test and the Wilcoxon rank sum test were used to compare continuous covariates between groups of patients who survived and died during the follow-up period. The chi-square test and Fisher's exact test were used to compare categorical and binary covariates between the two groups.

Linear mixed-effect models were used to compare the differences in BG levels and *Pi*-Ab levels among groups of patients who survived and died during the study period. The linear mixed-effect model is a regression technique for multiple observations for each individual to allow subsets of the regression parameters to vary randomly from one individual to another, thereby accounting for sources of natural heterogeneity of the patients. In addition, this regression method can overcome unbalanced data such as that produced when some patients passed away at 1.5 months. No additional BG and *Pi*-Ab levels from those patients were available for analysis beyond the 1.5-month mark. We ran the regression models to compare BG and *Pi*-Ab levels between the two groups for up to 3 months of the follow-up period. In this analysis, we used unstructured covariance matrix analysis in the regression models.

***In vitro* susceptibility testing.** *In vitro* susceptibility tests were performed with zoospores of *P. insidiosum*, isolated from patients with positive cultures, according to the CLSI M38-A2 protocol (28). All *P. insidiosum* isolates were confirmed by the PCR methods. *Candida parapsilosis* (ATCC 22019) and *Aspergillus flavus* (ATCC 204304) were used as controls. All isolates were tested against seven antifungal agents: amphotericin B, voriconazole (VRC), itraconazole (ITC), fluconazole, anidulafungin, caspofungin, and terbinafine (TRB). The MICs of these antifungal agents ranged from 0.125 to 64 mg/liter. Synergistic effects of VRC-TRB and ITC-TRB were tested by the checkerboard technique. The MICs of antifungal agents and combination drugs were interpreted in the unit of measurement of mg/liter and the fractional inhibitory concentration index (FICI) according to the following formula: $FICI = (MIC_{TRB\ in\ combination}/MIC_{TRB\ alone}) + (MIC_{VRC\ or\ ITC\ in\ combination}/MIC_{VRC\ or\ ITC\ alone})$, respectively. FICIs of ≤0.5, >0.5 to 4.0, and >4.0 indicated synergistic, indifferent, and antagonistic effect, respectively. A concentration of 0.05% carbendazim (Benomyl) was used as a positive control.

RESULTS

Patient characteristics. Fifty patients met the diagnostic criteria for vascular pythiosis and were recruited in this study: 22 proven vascular cases and 28 probable

TABLE 2 BG levels in sera of deceased and surviving patient groups[a]

Time (mo)	Mean (SD) BG level (pg/ml) in patient group		*P* value
	Deceased ($n = 5$)	Surviving ($n = 45$)	
0	498.2 (4.1)	482.8 (35.2)	0.33
0.5	500 (0)	387.7 (55.4)	<0.001
1.0	500 (0)	298.7 (46.9)	<0.001
1.5	484.4 (21.7)	154.4 (33.8)	<0.001
3.0	471.7 (24.7)[b]	80.7 (20.9)	<0.001

[a]BG, β-d-glucan.
[b]Values are for only three patients.

vascular cases. During the study period, 45 patients survived and 5 patients died. Baseline patient characteristics, treatment modalities, and posttreatment clinical information are summarized in Table 1. Definitive surgeries, defined by achievement of negative surgical margins, were achieved in all patients in both groups except one patient with a carotid lesion, in the deceased group, in which the definitive surgery was not possible. Three of the nonsurvivors underwent debridement to save their limbs. The duration of antifungal therapy in the deceased group was significantly shorter than that in the survival group (mean duration, 1.4 ± 0.9 months versus 5.9 ± 4.6 months; $P = 0.04$); however, this was because patients in the deceased group did not live long enough to complete the therapy.

Among the five patients who died, three were initially diagnosed with surgical wound infection; however, they were ultimately found to have residual pythiosis and died 3 months after enrollment. The patient with carotid disease decided to be on comfort measure only and not to pursue further work-up. The patient with femoral disease was further evaluated based upon the clinical symptoms of pain and erythema at the surgical site. None of these patients were under conditions that may have caused BG contamination (hemodialysis, received intravenous amoxicillin-clavulanate, albumin, or intravenous immunoglobulin) during the study period.

Serum BG levels among survivors and deceased patients. At the time of diagnosis, the mean (±standard deviation) BG levels in the survival group and the deceased group were not statistically different (482.8 ± 35.2 pg/ml versus 498.2 ± 4.1 pg/ml, respectively; $P = 0.33$) (Table 2). After the first dose of PIAI, patients in the survival group had significantly lower mean serum BG levels than the deceased group. Based on the recommended cutoff value, >80 pg/ml, for serum BG, all patients in this cohort had tested positive at the time of diagnosis, whereas all results from healthy and thalassemia patient donors (control group) were negative.

At each follow-up visit during the first 3 months, the mean serum BG levels significantly decreased among the survivor group and became negative after 3 months. However, the mean serum BG levels in the deceased group did not significantly change during the follow-up period (Table 2). In fact, the mean BG levels at 3 months remained highly positive until the patients died.

***P. insidiosum*-specific antibody among survivors and deceased patients.** By the in-house ELISA, the serum dilution of 1:800 presented the most significant difference between the upper and lower limits of OD_{490} values at each PIAI time point. Therefore, a dilution of 1:800 was used for the tested sera in this project.

At the time of diagnosis, all sera from vascular cases were positive for *Pi*-Ab, with a mean EV of *Pi*-Ab of 7.54 ± 1.8, whereas all negative-control sera presented negative results based on a cutoff EV of >1.5. The mean EVs of *Pi*-Ab for thalassemia patient donors and healthy donors were 1 ± 0.04 and 1 ± 0.03, respectively. The mean EV of *Pi*-Ab for patients with nocardiosis or other fungal infections was 1 ± 0.03, indicating no cross-reactivity with other infections. Given these results, the positive predictive value (PPV) and the negative predictive value (NPV) of *Pi*-Ab by the KCMH-ELISA were 100%.

Based on the results from the linear mixed-effect model (Table 3), the mean EV of *Pi*-Ab in the survival group was significantly higher than in the deceased group, at diagnosis (8.21 ± 0.7 and 2.43 ± 0.2, respectively; $P < 0.001$). At each follow-up visit,

TABLE 3 MICs of individual and combined agents against *P. insidiosum* isolates[a]

Isolate	Patient status	MIC of individual agents (mg/liter)							MIC of combined agents (mg/liter)		FICI[b] (interpretation)	MIC of combined agents (mg/liter)		FICI (interpretation)
		AMB	VRC	ITC	FLC	ANF	CAS	TRB	VRC	TRB		ITC	TRB	
1	Survived	4	2	1	1	2	2	2	1	2	1.5 (I)	1	2	2.0 (I)
2	Survived	4	4	2	2	4	4	4	2	2	1.0 (I)	2	2	1.5 (I)
3	Survived	8	2	2	4	8	2	2	2	2	2.0 (I)	2	2	2.0 (I)
4	Survived	4	4	1	2	8	4	2	2	2	1.5 (I)	0.5	2	1.5 (I)
5	Survived	8	2	4	8	8	4	4	2	4	2.0 (I)	2	4	1.5 (I)
6	Survived	4	2	2	4	8	4	2	1	2	1.5 (I)	1	2	1.5 (I)
7	Survived	4	4	4	4	4	2	4	2	4	1.5 (I)	1	2	1.5 (I)
8	Survived	4	2	2	2	4	2	2	1	1	1.0 (I)	2	2	2.0 (I)
9	Survived	4	4	2	2	2	4	2	2	2	1.5 (I)	0.5	1	1.0 (I)
10	Survived	8	8	2	2	4	8	4	4	4	1.5 (I)	2	4	2.0 (I)
11	Survived	4	2	1	4	4	2	2	1	2	1.5 (I)	1	2	2.0 (I)
12	Survived	8	4	2	4	4	2	2	2	2	1.5 (I)	1	2	2.0 (I)
13	Survived	4	2	2	2	4	2	4	2	2	1.5 (I)	1	2	1.5 (I)
14	Survived	4	4	2	2	4	4	4	2	2	1.0 (I)	2	2	1.5 (I)
15	Survived	4	2	4	2	8	4	2	2	2	2.0 (I)	2	2	1.5 (I)
16	Survived	4	4	4	2	4	4	4	2	4	1.5 (I)	4	4	2.0 (I)
17	Survived	4	4	2	4	4	2	2	2	2	1.5 (I)	2	2	2.0 (I)
18	Survived	8	4	2	4	2	4	4	1	4	1.25 (I)	1	4	1.5 (I)
19	Deceased	4	1	4	2	4	4	2	0.5	2	1.5 (I)	2	2	1.5 (I)
20	Deceased	4	2	4	4	8	2	4	2	4	2.0 (I)	4	4	2.0 (I)
21	Deceased	8	2	2	2	4	2	2	1	2	1.5 (I)	2	2	2.0 (I)
22	Deceased	4	4	1	4	4	2	2	2	2	1.5 (I)	1	2	2.0 (I)

[a]$n = 22$. Concentrations of tested antifungal agents ranged from 0.125 to 64 mg/liter. AMB, amphotericin B; VRC, voriconazole; ITC, itraconazole; FLC, fluconazole; ANF, anidulafungin; CAS, caspofungin; TRB, terbinafine.
[b]FICI, fractional inhibitory concentration index; I, indifferent.

there were no significant changes in EV of *Pi*-Ab levels among survivors. Patients in the survival group maintained an EV of *Pi*-Ab above 8 throughout the study period. The *Pi*-Ab levels among patients in the deceased group significantly increased by 0.28 per half month ($P = 0.02$). However, the average EV of *Pi*-Ab remained below 4 during their follow-up period.

***In vitro* susceptibility results.** The highest MICs were observed for AMB, ranging from 4 to 8 mg/liter, and the lowest MICs were observed for ITC, ranging from 1 to 4 mg/liter. No synergistic effect was found in the combination of either VRC-TRB or ITC-TRB, with a FICI ranging from 1.5 to 2.0 (Table 3).

DISCUSSION

We describe the first prospective cohort of vascular pythiosis patients who were followed up for 1 year after diagnosis. In our study, definitive surgery was performed in 49 of 50 (98%) patients. This is likely the reason why the mortality rate in our study decreased to 10% from 36.4% to 44.4% in the previous studies (13, 14, 29). As expected, patients who survived had a significant shorter mean duration from the onset of disease to their first medical encounters as well as a shorter mean waiting time for their definitive surgeries. This emphasizes the importance of early disease detection and prompt surgical intervention to improve survival. During the first 3 months of treatment course, only nonsurvivors developed fever, inflammation of the surgical sites, mass at the surgical sites, or arterial insufficiency syndrome. Unfortunately, these symptoms are not specific, and only one patient was further evaluated, based on these clinical symptoms, in a timely manner.

We found that all patients had high positive serum BG levels at diagnosis. Patients who survived had a significant decrease in BG levels at every visit. Conversely, patients who died had persistently high BG levels throughout the study period. These findings suggest that the trend in BG levels may be more important than the level itself. Vascular pythiosis patients with persistently elevated BG levels should be further evaluated for

any residual disease despite documented negative surgical margins from the pathology reports. The use of BG trends has been reported in previous studies of patients with candidemia (30) and *Pneumocystis jirovecii* pneumonia (31). As BG is not a *P. insidiosum*-specific biomarker, vascular pythiosis patients with persistent BG elevation should be evaluated for other possible causes of positive BG tests, including *Pseudomonas* infection, blood/albumin infusion, cellulose membranes in hemodialysis, and, in particular, intravenous amoxicillin-clavulanate, which is available in Thailand (32–34).

Similar to BG levels, all patients were *Pi*-Ab positive in this study at diagnosis based on a cutoff EV of >1.5. Interestingly, all patients in the survival group were able to maintain their EVs of *Pi*-Ab of >8 during the study period, whereas all patients in the deceased group had EVs of *Pi*-Ab of <4 throughout the study, despite the PIAI administration per protocol. We suspect that the persistent *Pi*-Ab in surviving patients, without evidence of ongoing infections for a year, is likely due to PIAI administration, and this phenomenon has been reported in the literature (16). These findings suggest that an EV of *Pi*-Ab of >8 may be a good prognostic indicator that implies a good host immune response to PIAI against pythiosis.

Importantly, our results have provided the opportunity to evaluate our treatment protocols. Our data suggest that aggressive surgery is still crucial, as the mortality rate in our study remained at 10% despite obtaining negative surgical margins in 98% of the cases. Likewise, we have learned that achievement of negative surgical margins does not necessarily mean that patients do not have residual infection. Complaints of any symptoms at the surgical site or proximal vascular lesions should be treated as ongoing vascular pythiosis until proven otherwise.

There are several limitations to this study. Pythiosis is a dangerous infection requiring aggressive therapy, and not all patients received exactly the same treatment. Accordingly, it was not possible to control all factors that might affect BG and *Pi*-Ab levels. In addition, since we are studying a relatively uncommon disease, a small sample size is unavoidable. Despite its small sample size, this is still the largest prospective study of vascular pythiosis to preliminarily evaluate BG and *Pi*-Ab levels as potential markers for disease monitoring. This study also adds *in vitro* susceptibility results from human isolates and describes contemporary clinical outcomes. Multicenter prospective studies are still required to determine how to incorporate BG and *Pi*-Ab levels into clinical decision-making. In addition, other nonspecific inflammatory markers, e.g., C-reactive protein and erythrocyte sedimentation rate, etc., should be investigated as potential lower-cost markers.

In summary, we believe that BG and *Pi*-Ab are potential markers in the management of vascular pythiosis and should be further investigated to determine whether their monitoring has an impact on clinical outcomes. Persistently elevated BG levels after a definitive surgery should prompt further evaluation for possible residual disease. A high EV of *Pi*-Ab may indicate a good host immune response to *P. insidiosum* and PIAI.

ACKNOWLEDGMENTS

This work was supported by the 90th and 100th Anniversary of Chulalongkorn University Scholarship, the National Research Council of Thailand (no. 406-699752-4 and FY 2017-Thesis Grant for Doctoral Degree Student), and the National Research Council of Thailand and Health Systems Research Institute (no. GRB_BSS_81_59_30_22). N.W., R.R., A.L., and A.C. received personal fees from the National Research Council of Thailand and Health Systems Research Institute (no. GRB_BSS_81_59_30_22).

The funders had no role in the study design, data collection and interpretation, or decision to submit the work for publication. M.F. is the Director of Clinical Development at Associates of Cape Cod, Inc. (Fungitell assay manufacturer). No other conflicts of interest are declared.

We thank all vascular surgeons, infectious disease specialists, and research coordinators at KCMH. We give special thanks to Supaporn Amornsirivat, Hematology Unit, Faculty of Medicine, Chulalongkorn University, for coordinating and supporting this project.

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