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Performance comparison of immunodiffusion, enzyme-linked immunosorbent assay, immunochromatography and hemagglutination for serodiagnosis of human pythiosis

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ABSTRACT

Pythiosis is a life-threatening infectious disease caused by the fungus-like organism *Pythium insidiosum*. Morbidity and mortality rates of pythiosis are high. The treatment of choice for pythiosis is surgical debridement of infected tissue. Early and accurate diagnosis is critical for effective treatment. In-house serodiagnostic tests, including immunodiffusion (ID), enzyme-linked immunosorbent assay (ELISA), immunochromatography (ICT) and hemagglutination (HA) have been developed to detect antibodies against *P. insidiosum* in sera. This study compares the diagnostic performance of ID, ELISA, ICT, and HA, using sera from 37 pythiosis patients and 248 control subjects. ICT and ELISA showed optimal diagnostic performance (100% sensitivity, specificity, positive predictive value and negative predictive value). ICT was both rapid and user-friendly. ELISA results were readily quantitated. ID is relatively insensitive. HA was rapid, but diagnostic performance was poor. Understanding the advantages offered by each assay facilitates selection of an assay that is circumstance-appropriate. This will promote earlier diagnoses and improved outcomes for patients with pythiosis.

1. Introduction

Pythiosis is a life-threatening infectious disease of humans and animals living in tropical and subtropical areas of the world (Mendoza et al., 1996). The etiologic agent is the fungus-like, pathogenic oomycete *Pythium insidiosum*. The natural habitat of *P. insidiosum* is wet lands including paddy fields, ponds and canals. *P. insidiosum* colonizes water plants, growing as a right-angle branching hyphae. It disseminates via motile biflagellate zoospores (an asexual stage) which act as the infectious agent (Mendoza et al., 1993).

While pythiosis in animals has been reported worldwide, the disease state in humans has been mostly restricted to Thailand (Mendoza et al., 1996; Krajaejun et al., 2006b). Physicians and clinical microbiologists remain largely unfamiliar with pythiosis and its causative agent. Patients usually present with one of the following clinical features (Sathapatayavongs et al., 1989; Krajaejun et al., 2006b): i) cutaneous pythiosis (granulomatous and ulcerating cutaneous lesions; accounting for 5% of all reported cases); ii) vascular pythiosis (claudication and gangrenous ulceration of extremities as a result of arterial occlusion; 59%); iii) ocular pythiosis (corneal ulcer or keratitis; 33%); or iv) infection of internal organs (3%). Diagnosis of pythiosis is difficult and often delayed. Use of conventional antifungal drugs for treatment of pythiosis is ineffective. The primary treatment option for pythiosis is the extensive surgical removal of infected tissue. Morbidity and mortality rates for pythiosis are high. Patients with vascular pythiosis require amputation of the infected extremity 80% of the time and 40% succumb to the infection. Eighty percent of patients with ocular pythiosis require enucleation to control disease progression.

It is hoped that early and accurate diagnosis minimizes the morbidity and mortality rates of pythiosis due to the critical importance of prompt treatment. In-house serodiagnostic tests, including immunodiffusion (ID) (Mendoza et al., 1986; Imwidthaya and Srimuang, 1989; Pracharktam et al., 1991), Western blot (WB) (Mendoza et al., 1992; Vanittanakom et al., 2004; Krajaejun et al.,

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2006a; Supabandhu et al., 2009), enzyme-linked immunosorbent assay (ELISA) (Mendoza et al., 1997; Grooters et al., 2002; Krajaejun et al., 2002; Vanittanakom et al., 2004), immunochromatographic test (ICT) (Krajaejun et al., 2009), and hemagglutination (HA) (Jindayok et al., 2009) have been developed to detect anti-*P. insidiosum* antibody in serum samples. Information on which serological assays provide optimal diagnostic performance is not currently available. Two types of antigen preparations are used for these assays: i) soluble antigen from broken hyphae (SABH) is used for ELISA (Mendoza et al., 1997; Grooters et al., 2002; Krajaejun et al., 2002) and WB (Mendoza et al., 1992; Krajaejun et al., 2006a); and ii) culture filtrate antigen (CFA) may be used for ID (Mendoza et al., 1986; Imwidthaya and Srimuang, 1989; Pracharktam et al., 1991), ELISA (Vanittanakom et al., 2004), WB (Vanittanakom et al., 2004; Supabandhu et al., 2009), ICT (Krajaejun et al., 2009) and HA (Jindayok et al., 2009). It was notable that SABH stored at room temperature underwent significant degradation compared to CFA stored similarly. Four investigators have published ELISA assays for diagnosis of pythiosis (Mendoza et al., 1997; Grooters et al., 2002; Krajaejun et al., 2002; Vanittanakom et al., 2004). Three of these used SABH antigen in their assays (Mendoza et al., 1997; Grooters et al., 2002; Krajaejun et al., 2002). In contrast, Vanittanakom et al., used CFA in their ELISA and only performed the assay using a single pythiosis serum specimen (Vanittanakom et al., 2004). The present study aims to: i) use CFA to develop an ELISA for comprehensive evaluation; and ii) compare performance of ELISA, ID, ICT, and HA for serodiagnosis of pythiosis.

2. Materials and methods

2.1. Serum samples

A total of 37 pythiosis serum samples were used in a performance comparison of serodiagnostic tests. These sera were collected from vascular (n = 32) and cutaneous (n = 5) pythiosis patients diagnosed based on at least one of the following criteria: i) successful isolation of *P. insidiosum* from infected tissue (n = 8) (Chaiprasert et al., 1990), or ii) successful detection of anti–*P. insidiosum* antibodies in serum samples (n = 37) by at least one of the following established serodiagnostic tests: ID, ELISA, HA, or ICT (Pracharktam et al., 1991; Krajaejun et al., 2002, 2009; Jindayok et al., 2009). Control samples (n = 248) included: i) 200 serum samples from healthy blood donors who came to the Blood Bank Division, Department of Pathology, Ramathibodi Hospital; ii) 10 serum samples from thalassemic patients without clinical evidence of pythiosis; iii) 13 serum samples from patients with positive antinuclear antibody (n = 10) or rheumatoid factor (n = 3); and iv) 25 serum samples from patients positive for other infectious diseases (aspergillosis, 3; zygomycosis, 2; candidiasis, 1; histoplasmosis, 1; cryptococcosis, 1; anti-human immunodeficiency virus antibody, 3; syphilis, 3; anti-hepatitis B virus, 7; and anti-hepatitis C virus, 4). Pooled positive and negative serum samples were prepared and used as an internal control in all assays. All serum samples were kept at −20 °C until used (the maximum length of time sera were stored at −20 °C prior to testing was ~5 years).

2.2. Antigen preparation

Crude extract antigen was prepared from the *P. insidiosum* strain Pi-S, using a previously described method (Jindayok et al., 2009; Krajaejun et al., 2009). The microorganism was isolated from a Thai patient with vascular pythiosis on July 13, 2009, and maintained on Sabouraud dextrose agar by subculturing once a month. Up to 10 small blocks containing actively growing mycelium (3-day culture) were transferred to a flask containing 200 mL of Sabouraud dextrose broth, shaken at 150 rpm and incubated at 37 °C for 10 days. Merthiolate was added to the culture at a final concentration of 0.02% (wt/vol). The culture was then filtered through a Durapore filter membrane (pore size, 0.22-μm). Phenylmethylsulfonyl fluoride (0.1 mg/mL) and EDTA (0.3 mg/mL) were added to minimize protein degradation in the filtered broth before it was concentrated ~80-fold using an Amicon 8400 apparatus with an Amicon Ultra-15 centrifugal filter (10,000 nominal molecular weight limit; Millipore, Bedford, MA). The concentrated broth was called CFA. Protein concentration was measured by spectrophotometer. CFA was kept at −20 °C until use.

2.3. ELISA

A 96-well polystyrene plate (Nunc, Roskilde, Denmark) was coated with 100 μL of CFA (5 μg/mL) in 0.1 mmol/L carbonate buffer (pH 9.6) at 4 °C overnight. The coated plate was then washed four times with phosphate buffer solution (pH 7.4) containing 0.05% Tween20 (PBS-T) and blocked with 1% casein at 37 °C for 30 min. In parallel with controls (pooled positive and negative sera), a test serum sample diluted 1:800 in 1% casein solution was added to each well (100 μL/well), incubated at 37 °C for 30 min and washed 4 times with PBS-T. Horseradish peroxidase-conjugated rabbit anti-human immunoglobulin G (Dako, Glostrup, Denmark) diluted 1:100,000 in 1% casein solution was added to each well (100 μL/well), incubated at 37 °C for 30 min, and washed four times with PBS-T. Freshly-prepared chromogen [1 mL of tetramethyl benzidine dihydrochloride (5 mg/mL) and 10 mL of hydrogen peroxide (0.1 mg/mL) in acetate buffer solution (25 μmol/mL)] was added to each well (100 μL/well) and incubated in a dark chamber at room temperature for 30 min. The enzymatic reaction was stopped with 100 μL of 0.5 N sulfuric acid. Optical density (OD) was measured using an ELISA reader (Behring Diagnostic) at the wavelengths 450 and 650 nm. Each serum sample was tested in duplicate. Mean OD value of each sample was corrected for the OD of the buffer control (1% casein solution). The OD value of each serum sample was divided by that of the same pooled negative control serum, and the derived value was referred to as ELISA value (EV).

2.4. ID

The ID test used in this study was modified from the method of (Pracharktam et al., 1991). Briefly, agar diffusion was carried out on a petri dish with 2% agar in Veronal buffer. CFA and serum sample were each added to 4-mm diameter wells located 4-mm apart. The pooled positive and negative serum controls were tested in the same manner against CFA. The petri dish was then incubated in a moist chamber for 24 h at room temperature. The presence of a precipitation line indicates a positive result.

2.5. ICT

ICT strip was prepared as described (Krajaejun et al., 2009). The ICT was tested in duplicate with 100 μL of diluted test or control serum (pooled positive or pooled negative) (1:10,000 in phosphate buffer, pH 7.4) in a 96-well polystyrene plate (Nunc, Roskilde, Denmark). Test and control ICT signals were read within 30 min. The result was considered positive when both test and control signals developed and negative when only the control developed.

2.6. HA

HA assay was prepared as described (Jindayok et al., 2009). Sheep red cells were coated with CFA. To perform HA, 25 μL of diluted serum (1:160 in 0.5% bovine serum albumin, 1% normal rabbit serum and 0.1% sodium azide in PBS) was added to each well of 96-well polystyrene plate (Nunc, Roskilde, Denmark). An equal volume of 0.5% CFA-coated sheep red cell suspension was added to each well and gently mixed. For the controls, the CFA-coated sheep red cell suspension was mixed with the pooled positive or negative serum. The plate was incubated for 1 h at room temperature. The presence of