รายงานวิจัยฉบับสมบูรณ์

โครงการ: การศึกษาทางชีววิทยาโมเลกุล พยาธิกำเนิด และภูมิคุ้มกันวิทยา ของเชื้อก่อโรคในคนชื่อ *Pythium insidiosum*

(Molecular Biology, Pathogenesis and Immunology Studies of the Human Pathogen *Pythium insidiosum*)

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มหาวิทยาลัยมหิดล

เดือน ปี ที่เสร็จโครงการ: ธันวาคม 2553

สัญญาเลขที่ RMU5080071

รายงานวิจัยฉบับสมบูรณ์

โครงการ

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สนับสนุนโดยสำนักงานคณะกรรมการการอุดมศึกษา
และสำนักงานกองทุนสนับสนุนการวิจัย

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ABSTRACT

The pathogenic oomycete *Pythium insidiosum* is a fungus-like microorganism that causes a life-threatening infectious disease, called pythiosis, in humans and animals living in tropic and subtropic countries. Thailand is an edemic area of human pythiosis. Basic information about *P. insidiosum*'s natural history is still very limited. New cases of pythiosis have been increasingly reported. Patients suffer from this disease and have poor prognosis. Diagnosis of pythiosis is difficult. Conventional antifungal drugs are ineffective for treatment. Surgical removal of infected organs (mostly, eyes and legs; leading to life-long disabilities) and death from progressive infection are common outcomes. Regarding diagnosis, there are needs for rapid, reliable, and easy-to-use diagnostic tests. Regarding treatment, it is apparent that more needs to be done in the way of basic research to provide insights into *P. insidiosum*'s biology and pathogenesis, and thereby lead to the discovery of novel strategies for pathogen and infection control, such as, new antimicrobial agents and vaccines. Here, we report two newly-developed serodiagnostic tests (user-friendly formats of immunochromatography and hemagglutination) for facilitating rapid and reliable diagnosis of human pythiosis. The tests have high detection specificity and sensitivity, and a short turn around time of 30-60 minutes. We also report a genetic identification and characterization of the 74-kDa immunodominant protein of *P. insidiosum*, which is a potential virulence factor, diagnostic target, and vaccine candidate. The 74-kDa immunodominant antigen is encoded by a putative exo-1,3-beta glucanase gene. Glucanase genes are an interesting group of genes since in some pathogenic fungi, they have roles play in pathogenesis (adhesion and invasion) and host immune responses (stimulation of host immunity). Based on the glucanase gene, 22 *P. insidiosum* clinical isolates are divided into 2 phylogenetic groups. Because genetic and genomic information is lacking for *P. insidiosum*, which makes molecular studies of this pathogen difficult, we have undertaken an EST study, and report on the first dataset of 486 ESTs, assembled into 217 unigenes. Of these, 144 had significant sequence similarity with know genes, including 47 with ribosomal protein homology. Potential virulence factors included genes involved in antioxidation, thermal adaptation, immunomodulation, and iron and sterol binding. Effectors resembling pathogenicity factors of plant-pathogenic oomycetes, were also discovered. In conclusion, our project, supported by the Thailand research fund and the commission on higher educationhave, as a final report, has successfully developed two useful tests for rapid diagnosis of human pythiosis, shown that the 74-kDa immungen is glucanase (a potential candidate for diagnostic test and vaccine development), and reported the first EST dataset of *P. insidiosum* for exploring genetic diversity and better understanding pathogenesis of the pathogen.

บทคัดย่อ

Pythium insidiosum คือ fungus-like microorganism ที่อยู่ในกลุ่ม oomycetes และเป็นสาเหตุของโรคติดเชื้อร้ายแรงที่เรียกว่า “Pythiosis”. โรคนี้เกิดได้ในคนและสัตว์ที่อาศัยอยู่ในประเทศเขตร้อน. ประเทศไทยถือเป็น endemic area ของโรค pythiosis ที่เกิดในคน. ในปัจจุบัน มีการรายงานการพบโรค pythiosis มากขึ้น. อย่างไรก็ตาม ความรู้ความเข้าใจเกี่ยวกับโรคนี้ยังมีไม่มากนัก. ผู้ป่วยเกือบทั้งหมดจะมีการพยากรณ์โรคไม่ดี. การวินิจฉัยมีความยากลำบาก. การรักษาด้วยราต้านเชื้อราไม่ได้ผล การรักษาหลักจึงเป็นการผ่าตัดส่วนที่ติดเชื้อออก (อวัยวะที่ติดเชื้อมากที่สุดคือตาและขา) ซึ่งทำให้เกิดความพิการถาวร และในบางรายการที่โรคลุกลามไปมาก ผู้ป่วยจะเสียชีวิตจากการติดเชื้อในที่สุด. เมื่อพิจารณาถึงปัญหาการวินิจฉัย พบว่ามีความต้องการชุดทดสอบที่มีประสิทธิภาพ ให้ผลรวดเร็ว และสามารถใช้ได้ง่าย. และหากพิจารณาถึงปัญหาการรักษา พบว่ามีความจำเป็นที่ต้องศึกษาด้านวิทยาศาสตร์พื้นฐาน เพื่อค้นหาวิธีการรักษาใหม่ที่ได้ผลดีกว่าเดิม เช่น ยาหรือวัคซีนต้านเชื้อ *Pythium*. ในโครงการวิจัยนี้ คณะผู้วิจัยได้พัฒนาชุดตรวจเลือดเพื่อช่วยวินิจฉัยโรค human pythiosis สองวิธี คือ immunochromatography และ hemagglutination ซึ่งมีหลักการทดสอบที่ง่ายต่อการใช้งาน มีประสิทธิภาพสูง และให้ผลที่รวดเร็ว (เวลาที่ใช้ในการทดสอบ ~30-60 นาที). คณะผู้วิจัยยังได้รายงานการศึกษาและวิเคราะห์ทางชีวพันธุศาสตร์ ซึ่งพบว่า immunodominant protein ขนาด 74-kDalton ของเชื้อ *P. insidiosum* คือ เอนไซม์ exo-1,3-beta glucanase gene ซึ่งอาจสามารถนำมาพัฒนาเป็น vaccine ได้. Glucanase genes เป็นโปรตีนที่น่าสนใจ เพราะในเชื้อราบางชนิด glucanase มีบทบาทในการก่อให้เกิดโรค (ช่วยให้เกิดการเกาะติดของเชื้อ และช่วยในการชอนไชเนื้อเยื่อ) และมีบทบาทในการกระตุ้นภูมิกันของร่างกาย. จากการใช้ glucanase gene เพื่อศึกษา phylogenetics พบว่าเชื้อ *P. insidiosum* ที่แยกได้จากผู้ป่วยจำนวน 22 สายพันธุ์ ถูกแบ่งได้เป็น 2 กลุ่ม. นอกจากนี้คณะผู้วิจัยยังได้ศึกษาความหลากหลายของยีนของ *P. insidiosum* โดยวิธี expressed sequence tags (ESTs) เพราะข้อมูลทางชีวพันธุศาสตร์ของเชื้อ *P. insidiosum* มีน้อยมาก ทำให้เป็นอุปสรรคต่อการศึกษาด้าน molecular pathogenesis. ในการศึกษานี้ คณะผู้วิจัยรายงาน ESTs จำนวนทั้งสิ้น 486 ESTs ซึ่งสามารถ assembled ได้ 217 unique genes. ในจำนวนนี้ 144 ESTs มีลำดับเบสคล้ายกับยีนของสิ่งมีชีวิตอื่นที่รายงานในฐานข้อมูลสากล. แต่ที่น่าสนใจคือ พบมี ESTs จำนวนหนึ่งมีลำดับเบสคล้ายกับ virulence factors ของเชื้อก่อโรคชนิดอื่น เช่น ยีนที่มีบทบาทเกี่ยวกับ antioxidation, thermal adaptation, immunomodulation, และ iron and sterol binding. นอกจากนี้ยังพบ effector genes ซึ่งน่าจะมีบทบาทในการก่อให้เกิดโรคด้วย. โดยสรุปแล้วโครงการวิจัยนี้ (ซึ่งได้รับทุนจาก สกว-สกอ) ประสบความสำเร็จในการพัฒนาชุดตรวจวินิจฉัยโรค human pythiosis, ค้นหาและวิเคราะห์ยีนที่ถอดรหัสเป็น 74-kDa immunogen, และรายงานฐานข้อมูลชีวพันธุศาสตร์ของเชื้อ *P. insidiosum* โดยใช้ ESTs เพื่อเป็นจุดเริ่มต้นให้เข้าใจชีววิทยาและกระบวนการเกิดโรค pythiosis.

หน้าสรุปโครงการ (EXECUTIVE SUMMARY)

1. ชื่อโครงการ

การศึกษาทางชีววิทยาโมเลกุล พยาธิกำเนิด และภูมิคุ้มกันวิทยา ของเชื้อก่อโรคในคนชื่อ *Pythium insidiosum*

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โครงการย่อยที่ 1: Identification, characterization, and clinical applications of a gene encoding an immunodominant antigen of the human pathogen *Pythium insidiosum*

โครงการย่อยที่ 2: Initial screening for gene diversity in the human pathogen *Pythium insidiosum*

โครงการย่อยที่ 3A & 3B: Development of a rapid serological test for diagnosis of human pythiosis

2. ชื่อหัวหน้าโครงการ

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3. สาขาวิชาที่ทำวิจัย: สาขาวิทยาศาสตร์การแพทย์และเทคโนโลยีชีวภาพ

4. ระยะเวลาดำเนินงาน: 3 ปี 6 เดือน (มิถุนายน 2550 – ธันวาคม 2553)

5. ปัญหาที่ทำวิจัย และความสำคัญของปัญหา

โรค **pythiosis** เป็นโรคติดเชื้ออุบัติใหม่ (emerging infectious disease) เกิดจากเชื้อ ***Pythium insidiosum*** ซึ่งเป็นเชื้อในกลุ่ม **oomycetes**. โรคนี้มีความรุนแรงถึงขั้นเสียชีวิต. พบได้ทั้งในคนและสัตว์ในประเทศเขตร้อน. *P. insidiosum* สามารถสร้าง zoospore ซึ่งสามารถว่ายน้ำไปเกาะผิวหนังหรือเยื่อบุต่างๆ และชอนไชไปสู่เนื้อเยื่อที่อยู่ลึกลงไป ก่อให้เกิดพยาธิสภาพ. ผู้ป่วย human pythiosis พบได้ในประเทศไทยเป็นส่วนใหญ่. จึงถือได้ว่าประเทศไทยเป็น **endemic area** ของโรคนี้. ลักษณะทางคลินิกของโรค pythiosis สามารถแบ่งได้เป็น 4 กลุ่ม คือ (1) ติดเชื้อที่ผิวหนัง (cutaneous/subcutaneous form) พบ 5% ของผู้ป่วยทั้งหมด ผู้ป่วยมักมาด้วยแผลเรื้อรังที่ชั้นผิวหนังและใต้ผิวหนัง สามารถรักษาได้ด้วยยา SSKI, (2) ติดเชื้อที่เส้นเลือดแดง (vascular form) พบ 59% ของผู้ป่วยทั้งหมด ผู้ป่วยมักมาด้วย claudication หรือ gangrene ที่ขา ส่วนใหญ่รักษาโดยการตัดขาข้างที่ติดเชื้อทิ้ง ในรายที่มีการติดเชื้อรุกลามมากก็อาจเสียชีวิตได้จากการแตกของหลอดเลือดแดงใหญ่, (3) ติดเชื้อที่ตา (ocular form) พบ 33% ของผู้ป่วยทั้งหมด ผู้ป่วยมักมาด้วยแผลที่กระจกตา ส่วนใหญ่ต้องรักษาโดยการผ่าตัดนำลูกตาข้างที่ติดเชื้อออก, และ (4) ติดเชื้อบริเวณอื่น (disseminated form) พบ 3% ของผู้ป่วยทั้งหมด ผู้ป่วยในกลุ่มนี้พบติดเชื้อที่เนื้อสมอง ที่โพรงจมูกและไซนัส หรือที่กระเพาะและลำไส้ ผู้ป่วยทั้งหมดในกลุ่มนี้เสียชีวิต. จากการศึกษาทางระบาดวิทยา พบมีผู้ป่วยกระจายอยู่ทั่วประเทศ. มีปัจจัยที่สัมพันธ์กับผู้ป่วยโรค pythiosis เช่น โรค **thalassemia**, การทำอาชีพเกษตรกรรม, ช่วงอายุ 20-60 ปี, และเพศชาย.

ปัญหาสำคัญของโรค pythiosis คือ เป็นโรคที่มีอัตราทุพพลภาพและอัตราการตายสูง เนื่องจากบุคลากรทางการแพทย์ไม่รู้จักโรค; ขาด diagnostic test ที่ให้ผลรวดเร็ว ง่ายต่อการใช้ และมี sensitivity/specificity ที่ดี เพื่อใช้ในชนบทห่างไกลที่พบผู้ป่วยมาก; และการรักษาทางยาที่มีอยู่ในปัจจุบันใช้ไม่ได้ผล วัคซีนที่ใช้รักษาโรคมีประสิทธิภาพต่ำ การรักษาหลักจึงต้องใช้การผ่าตัดเนื้อเยื่อหรืออวัยวะที่ติดเชื้อออกให้หมดซึ่งทำให้เกิดความพิการ. ด้วยเหตุนี้ งานวิจัยที่ข้าพเจ้าและคณะฯ ต้องการมุ่งเน้น คือ พัฒนา rapid and convenient diagnostic tests ที่มีประสิทธิภาพมากขึ้น เพื่อช่วยวินิจฉัยโรคในชนบท (โครงการย่อยที่ 3); ศึกษาหา vaccine candidate เพื่อนำมาพัฒนาเป็น immunotherapeutic vaccine ที่มีประสิทธิภาพมากขึ้น (โครงการย่อยที่ 1). นอกเหนือจากนี้ การทำวิจัยพื้นฐานโรค human pythiosis (โครงการย่อยที่ 1 และ 2) ก็สำคัญและจำเป็น เพราะจะทำให้เราเข้าใจ natural history, pathogenesis และ host responses เพื่อสร้างองค์ความรู้ใหม่ที่เป็นพื้นฐานต่อการพัฒนาต่อไปได้.

6. วัตถุประสงค์

โครงการย่อยที่ 1: เพื่อ identify และ characterize หน้าที่ของยีนที่ถอดรหัสเป็น immunodominant antigen ของเชื้อ *P. insidiosum* และเพื่อประยุกต์ใช้ยีนที่พบ สำหรับศึกษา molecular typing และ phylogeny ของเชื้อ *P. insidiosum* ที่พบในประเทศไทย และหาความสัมพันธ์ระหว่างความแตกต่างทางสายพันธ์ กับลักษณะทางคลินิก หรือ geographic distribution ของเชื้อ.

โครงการย่อยที่ 2: เพื่อจัดทำ expressed sequence tag (EST) database และศึกษา gene diversity ของเชื้อ *P. insidiosum* และเพื่อศึกษา function ของยีนใน *P. insidiosum* EST database ที่มีความน่าสนใจทาง biology และ pathogenesis

โครงการย่อยที่ 3: เพื่อพัฒนาชุดตรวจเลือด สำหรับช่วยวินิจฉัยโรค human pythiosis ที่รวดเร็ว มีประสิทธิภาพและสามารถนำไปใช้ในพื้นที่ห่างไกลได้ เช่น agglutination test และ immunochromatography

8. ความเชื่อมโยงกับต่างประเทศ

- Dr. Thomas D. Sullivan, PhD (University of Wisconsin-Madison, WI, USA) ให้คำปรึกษา สนับสนุนเทคโนโลยีอุปกรณ์ (Mass spectrometry), research materials และ review manuscript.

- Dr. Vipaporn Phuntumart, PhD (Bolwing Green State University, OH, USA) ให้คำปรึกษา ร่วมมือวิเคราะห์ *P. insidiosum* ESTs และ review manuscript.

เนื้อหางานวิจัย

<u>โครงการย่อยที่ 1</u>: Identification, characterization, and clinical applications of a gene encoding an immunodominant antigen of the human pathogen *Pythium insidiosum*

<u>วัตถุประสงค์</u>: เพื่อ identify และ characterize หน้าที่ของยีนที่ถอดรหัสเป็น immunodominant antigen ของเชื้อ *P. insidiosum* และ เพื่อประยุกต์ใช้ยีนที่พบ สำหรับศึกษา molecular typing และ phylogeny ของเชื้อ *P. insidiosum* ที่พบในประเทศไทย และหาความสัมพันธ์ระหว่างความแตกต่างทางสายพันธ์ กับลักษณะทางคลินิก หรือ geographic distribution ของเชื้อ.

<u>วิธีการวิจัย</u>

Microorganisms and antigen preparations: Twenty-two clinical isolates of *Pythium insidiosum* used in this study, and clinical details, including the form of pythiosis and the geographic origin of patients for each isolate, are listed in Table 1. Antigen preparation was as previously described (18). Briefly, *P. insidiosum* strain P1 was cultured on Sabouraud dextrose agar for 3 days at 37°C. Ten small agar pieces, containing hyphal elements from the growing culture, were transferred to 100 ml of Sabouraud dextrose broth and shaken (150 rpm) for 10 days at 37°C. Merthiolate (0.02% [wt/vol]) was added to kill the cultures before they were filtered through a Durapore membrane filter (0.22-μm pore size; Millipore, County Cork, Ireland). The filtered broth containing phenylmethylsulfonyl fluoride (PMSF; 0.1 mg/ml) and EDTA (0.3 mg/ml) is referred to as culture filtrate antigen (CFA). The retained hyphal mass was transferred to a mortar in the presence of 25 ml of cold sterile distilled water containing PMSF (0.1 mg/ml) and EDTA (0.3 mg/ml). The hyphal mass was ground on ice and centrifuged at 4,800 x *g*, for 10 min at 4°C. The resulting supernatant, referred to as soluble antigen from broken hyphae (SABH), was filtered through a Durapore membrane filter (0.22-μm pore). SABH and CFA were concentrated ~80-fold using an Amicon Ultra 15 centrifugal filter (10,000 nominal molecular weight limit; Millipore, Bedford, MA). The concentrated preparations were stored at -20°C until use.

Serum samples: Sera from 5 patients with vascular pythiosis (T1-T5; diagnosed by culture identification and serological tests) and 5 healthy blood donors (C1-C5) who came to the Blood Bank Division, Ramathibodi Hospital were used for evaluating immunoreactivity of synthetic peptides in this study. All sera were stored at -20°C until use.

SDS-PAGE and Western blot: SDS-PAGE and Western blot analysis were performed according to the procedures previously described (18). Briefly, ~15 μg of SABH or CFA was mixed with loading buffer (60 mM Tris-HCl, pH 6.8, 25% glycerol, 2% sodium dodecyl sulfate [SDS], 14.4 mM mercaptoethanol, and 0.1% bromophenol blue), boiled for 5 min, and centrifuged at 14,000 x *g* for 5 min. Supernatant proteins were separated at 100 V in an SDS-polyacrylamide gel (4% stacking gel, 12% separating gel) on a minigel apparatus (Bio-Rad, Hercules, CA). The gel was stained with Coomassie brilliant blue. Prestained SDS-PAGE molecular weight standards (Bio-Rad) were run in parallel.

For Western blot analysis, separated antigens were electro-transferred from SDS-PAGE gels to polyvinylidene difluoride membranes (Bio-Rad) for 1 h at 100 V. Membranes were blocked with 5%

gelatin in Tris-buffered saline (TBS), pH 7.5, and incubated overnight at room temperature with a pythiosis serum T1 diluted at 1:1,000 with antibody buffer (TBS, pH 7.5, 0.05% Tween 20, 1% gelatin). The membranes were washed twice with washing buffer (TBS, pH 7.5, 0.05% Tween 20) and incubated at room temperature for 2 h with goat anti-human immunoglobulin G (heavy plus light chains) conjugated with horseradish peroxidase (Bio-Rad) at a 1:3,000 dilution with antibody buffer. The membranes were washed with washing buffer three times. Signals were developed by adding a fresh mixture of 10 ml of 0.3% 4-chloro-1-naphthol in methanol and 50 ml of TBS with 30 µl of 30% H_2O_2. Reactions were stopped by immersing the membranes in distilled water. Color developed membranes were immediately photographed.

Mass spectrometric analysis: SABH and CFA protein bands corresponding to the 43- and 74-kDa immunonodominant antigens, separated by one-dimension SDS-PAGE, were excised from a Coomassie brilliant blue-stained gel, and cut into 1-mm pieces. Destaining, trypsin in-gel digestion, extraction and peptide purification were performed using the protocol of Saveliev et al. (37). The peptides were subjected to analysis on the MDS SCIEX 4800 MALDI-Tof/Tof mass spectrometer (MS; Applied Biosystems, Foster City, CA, USA) at the Biotechnology Center, University of Wisconsin-Madison. Peptide mass fingerprints (PMF) were generated and used to match to theoretical trypsin-digested proteins in the National Center for Biotechnology Information (NCBI) non-redundant database using the program MASCOT (37). Some peptide masses were selected for generating *de novo* amino acid sequence tags by MALDI-Tof/Tof tandem mass spectrometric (MS/MS) analysis. The *de novo* amino acid sequence of each peptide was BLASTed against the NCBI non-redundant protein database (blastp).

Genomic DNA extraction, PCR and DNA sequencing: Genomic DNA extraction was performed using the protocol of Pannanusorn et al. (31) or Vanittanakom et al. (43). The 1.1-kb region of the *P. insidiosum* ß-glucanase gene sequence was PCR amplified using degenerate primer Pr1 (5'-ACNTTYGARCAYTGGCCNAT-3') and Pr4 (5'-ARRTGCCARTAYTGRTTRAA-3'). Alternatively, Pr7 (5'-ACGTTCGAGCACTGGCCTAT-3') and Pr10 (5'-AGGTGCCAGTACTGGTTGAA-3') (which are subset primers of Pr1 and Pr4, respectively, that were derived from the sequence of the putative exo-1,3-ß glucanase gene *EXO1* of *Phytophthora infestans*) were used to amplify glucanase DNA sequence of some *P. insidiosum* isolates. The amplifications were carried out in 50-µl, containing 200 ng genomic DNA, 0.2 mM dNTPs, 1 µM of each primer, and 1 µl of Elongase DNA polymerase mixture (Invitrogen) in 1x Elongase buffer (buffer A:B = 1:4). Amplifications were performed in a MyCycler Thermal Cycler (Bio-Rad, USA), with the following cycling temperatures: denaturation at 94^{O}C for 2.5 min for the first cycle and 30 sec for subsequent cycles, annealing for 30 sec at 40^{O}C and elongation for 1.5 min at 68^{O}C for a total of 35 cycles, followed by a final extension for 10 min at 68^{O}C. The PCR products were assessed for amount and size by 1% agarose gel electrophoresis.

Direct sequencing of PCR products was performed using the Big Dye Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems, USA). Primer Pr1, Pr7 and Pr46 (5'-ACCTCATGTCCAAGAAGCTCAACG-3') were used to obtain the forward sequences and primer Pr4, Pr10 and Pr43 (5'-CGCGCATAAAGTCGAGCCAGAA-3') were used for the reverse sequence. Automated sequencing was carried out using an ABI 3100 Genetic Analyzer (Applied Biosystems, USA) and analyzed using the Applied Biosystems Sequencing software.

Synthetic peptides and ELISA: s74-1 (WTSIASTQPVGTTTFEHWPIR) and s74-2 (FLTLEEQCDWAFNQYWHLNR) synthetic peptides (≥ 95% pure) were purchased from BioSynthesis Inc. (Lewisville, Texas, USA). Each peptide was dissolved in sterile distilled water. The s74-1 and s74-2 peptides were diluted to a final concentration of 0.1 µg/ml in 50 mM phosphate buffer with 0.6 M NaCl (pH 5.6) or 50 mM acetate buffer with 0.6 M NaCl (pH 2.6). Each peptide was used to coat a 96-well polystyrene plate (COSTAR, Corning Incorporated, New York) (100 µl/well) at 4 °C, overnight. Unbound antigens were removed by washing 4 times with washing buffer (0.1M Tris-NaCl buffer with 0.1% Tween). Serum samples from pythiosis patients and blood donors were 200-fold diluted in 0.15 M PBS (pH 7.2) with 1% BSA (PBS-BSA), added to wells in triplicate (100µl/well), incubated at 37 °C for 30 min and washed 4 times. Peroxidase-conjugated goat anti-human IgG Fc antibody (AffiniPure, Jackson ImmunoResearch Laboratories Inc.) was diluted 100,000-fold in PBS-BSA, added to each well (100µl/well), and incubated at 37 °C for 30 min. After washing unbound conjugate antibody 4 times, 100 µl of the TMB solution (the galactomannan ELISA kit, Bio-Rad) was added to each well. The reaction was stopped by adding 100 µl of 1.5 N H_2SO_4 to each well. The optical density was measured with an ELISA reader (Behring Diagnostic) at 450 and 650 nm.

Phylogenetic analysis: The putative exo-1,3-ß glucanase partial gene sequences of all *P. insidiosum* isolates (Table 1) were aligned by using the Clustal W program (11) and were manually edited. The phylogenic tree of the *P. insidiosum* glucanase gene sequences, and the *P. infestans EXO1* sequence (set as the outgroup for rooting) was generated by neighbor-joining (NJ) algorithm in the Phylip program, and drawn using the Treeview program (30). The reliability of the inferred trees was tested using bootstrap re-sampling with 1,000 replicates (7).

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Mass spectrometric analysis revealed 74-kDa antigen is ß-glucanase: Two abundant antigenic proteins, namely s74 and c74, corresponding to the 74-kDa major immunodominant bands of SABH and CFA, respectively (18), were excised from a one-dimension SDS-PAGE gel to use for peptide mass fingerprinting (PMF) by mass spectrometric analysis following trypsin digestion. Comparison of the PMFs of s74 and c74 antigens did not reveal any significant spectrum similarity (Fig. 1). Peptide masses of each PMF were used to search theoretical trypsin-digested proteins in the NCBI non-redundant database and two peptide masses of the s74 antigen, 2415.3 (relative intensity, 34%) and 2671.4 (relative intensity, 3%), matched to 2 peptides, WTSIASTQPVGTTTFEHWPIR (= s74-1 peptide) and FLTLEEQCDWAFNQYWHLNR (= s74-2 peptide), of a putative exo-1,3-ß glucanase (EXO1) from the plant-pathogenic oomycete *P. infestans* (accession number AAM18483) (Fig. 1). The s74-1 and s74-2 peptides were matched to amino acid positions 278-298 and 634-653 of *P. infestans* EXO1, respectively. The *de novo* sequence of the s74-1 peptide determined by MS/MS analysis showed the same amino acid sequence as predicted by the initial MS analysis. For the PMFs generated for the c74 protein, no peptide mass matched to any protein in the NCBI database. Some of the c74 peptide masses with strong relative intensity (i.e., 1109.6, 1155.5, 1238.6, 1281.6, 1564.8 and 2154.1) underwent *de novo* sequencing by MS/MS analysis. The *de novo* amino acid sequences obtained (Table 2) were subjected to BLAST of the NCBI database, but none of them matched to any protein. Another abundant protein, namely s43, corresponding to the 43-kDa immunodominant band of SABH (18) was isolated from the

SDS-PAGE gel, and was subjected to the MS and MS/MS analyses. Neither PMF of this peptides nor *de novo* sequences of selected peptide masses with strong intensity (i.e., 1842.8, 1859.8 and 2153.0; Table 1) matched to any protein in the NCBI database.

Partial DNA sequence of the *P. insidiosum* ß-glucanase gene: PCR primers were designed from within the reverse translated amino acid sequences of the s74-1 and s74-2 peptides. Degenerate primer Pr1 (128-fold degeneracy) and Pr4 (64-fold degeneracy) were used to amplify genomic DNA of *P. insidiosum* strains P17, P24 and P40 (Table 1), generating an intense 1.1-kb fragment. DNA sequences of the 1.1-kb PCR product of each strain were analyzed by BLAST of the NCBI nucleotide database (blastn). The highest blastn match (identity, 71%; E-value, < 1e-146) was to a putative exo-1,3-ß glucanase gene of *P. infestans* (*EXO1*; accession number AF494014). The deduced amino acid sequence (363 amino acids) of the partial putative exo-1,3-ß glucanase gene of *P. insidiosum* amplified from strain P17 had in-frame translations with the s74-1 and s74-2 peptides (Fig. 2A). The amino acid sequence deduced from the PCR product, fused with the complete s74-1 and s74-2 peptide sequences (all together 379 amino acids) was used to BLAST against the NCBI protein database (blastp) and the best match (identity, 72% (276/379); positivity, 83% (315/379); gap, 0.8% (3/379); E-value, 8e-170) was a exo-1,3-ß glucanase protein of *P. infestans* (EXO1; accession number AAM18483) (Fig. 2A). An NCBI Conserved Domain Database search (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) revealed two conserved domains, BglC and the X8 superfamily (Fig. 2B). Similar results were observed for *P. insidiosum* strain P24 and P40.

Immunoreactivity of *P. insidiosum* ß-glucanase peptides: Synthetic s74-1 and s74-2 peptides were each used to coat ELISA plates and tested against pythiosis (T1-T5) and control (C1-C5) serum samples. Among pythiosis sera, T1 (Hemagglutination (HA) titer (12) = 1:5,120) and T5 (HA titer = 1:40,960) pythiosis sera gave remarkably high ELISA signals (1.2 and 0.8, respectively, for s74-1; 1.2 and 1.0, respectively, for s74-2), while T2 (HA titer = 1:2,560), T3 (HA titer = 1:1,280) and T4 (HA titer = 1:1,280) pythiosis sera gave weak to moderate ELISA signals (0.4, 0.3 and 0.3, respectively, for s74-1; 0.1, 0.2 and 0.2, respectively, for s74-2). In general, the s74-1 (Fig. 3A) or the s74-2 (Fig. 3B) peptide gave similar ELISA signal patterns with the different sera.

For the s74-1 peptide, the mean ELISA signal of pythiosis sera was 0.48 (range, 0.26-1.17; SD, 0.39), while the mean ELISA signal of control sera was 0.15 (range, 0.07-0.27; SD, 0.09). If a cutoff point of 0.32 is set, which equals the mean ELISA signal of control sera plus two standard deviations of the mean (2SDs), 60% of pythiosis sera had ELISA signals were above this cutoff, but 100% of the control sera were below the cutoff (Fig. 3A). For the s74-2 peptide, the mean ELISA signal of the pythiosis sera was 0.57 (range, 0.33-1.22; SD, 0.39), while mean ELISA signal of the control sera was 0.09 (range, 0.05-0.18; SD, 0.06). Based on the cutoff point of 0.21 (the mean ELISA signal of control sera plus 2SDs), all pythiosis sera were clearly differentiated from all control sera (Fig. 3B).

Phylogenetic analysis of *P. insidiosum* ß-glucanase genetic sequences: ß-glucanase gene sequences of 22 *P. insidiosum* isolates from patients with different forms of pythiosis, who lived in different regions of Thailand (Table 1), were successfully amplified and sequenced using primers Pr1 and Pr4, or Pr7 and Pr10. These sequences were analyzed for phylogenetic relatedness using the NJ algorithm, (setting the *P. infestans EXO1* as an outgroup for rooting). From this analysis, *P. insidiosum* can be divided into two phylogenetic groups (clade-A and clade-B) with 98% and 100% reliability (Fig.

4). Distribution of the forms of pythiosis and the geographic origins of the patients in the two clades were similar.

Note: Please see the tables, figures, and references in the published version in the appendix

<u>โครงการย่อยที่ 2</u>: Initial screening for gene diversity in the human pathogen *Pythium insidiosum*

<u>วัตถุประสงค์</u>: เพื่อจัดทำ expressed sequence tag (EST) database และศึกษา gene diversity ของเชื้อ *P. insidiosum* และเพื่อศึกษา function ของยีนใน *P. insidiosum* EST database ที่มีความน่าสนใจทาง biology และ pathogenesis

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Strain, growth condition and RNA extraction

The *P. insidiosum* strain CBS119452, isolated from a Thai patient with vascular pythiosis, was used to prepare RNA. The microorganism was cultured in a Petri dish containing 10 ml of Sabouraud dextrose broth and incubated at 37 °C for 5 days. Two loops full of growing mycelium were disrupted with glass beads (diameter, 710-1,180 µm; Sigma, St. Louis, MO), using TissueLyzer MM301 (Qiagen, Germany). Total RNA was extracted using Trizol Reagents (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. The RNA concentration and purity were measured using spectrophotometry (UV-1700, Shimadzu Corp., Japan), and the integrity was evaluated by agarose gel electrophoresis. The RNA was stored at -80 °C until use.

Construction of cDNA library

Full-length cDNAs of *P. insidiosum* were generated from 3.5 µg of total RNA, using the GeneRacer kit (Invitrogen) according to the manufacturer's protocol. Using 0.5 µl of freshly-prepared cDNAs as template, they were amplified with 0.025 U of proof-reading Platinum *Pfx* DNA polymerase (Invitrogen), in 1x *Pfx* amplification buffer with 1 mM of $MgSO_4$, 0.3 mM each of dNTPs, 0.9 µM each of the GeneRacer 5' primer (5′-CGACTGGAGCACGAGGACACTGA-3′) and the GeneRacer 3' primer (5′-GCTGTCAACGATACGCTACGTAACG-3′), in a MyCycler thermal cycler (Bio-Rad, Hercules, CA) with the following cycling temperatures: an initial denaturation at 94 °C for 2 min, and a total of 35 amplification cycles: 5 cycles of denaturation for 30 s at 94 °C and annealing and elongation for 3 min at 72 °C, 5 cycles of denaturation for 30 s at 94 °C and annealing and elongation for 3 min at 70 °C, 25 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at 60 °C and elongation for 3 min at 68 °C, and followed by a final extension for 10 min at 68°C. The PCR products were assessed for the expected heterogeneous-sized bands by 1% agarose gel electrophoresis. The PCR products were inserted to the pCR4-blunt-TOPO vector using the Zero Blunt TOPO PCR cloning kit (Invitrogen) and then transformed to the electrocompetent *Escherichia coli* strain DH5α, according to the manufacturer's protocol. The transformed bacteria were incubated overnight on LB agar containing 100 µg of ampicillin.

Colony PCR and DNA sequencing

A small portion of a transformed bacterial colony was suspended in 5 µl sterile water (to serve as PCR template) and was all added to a 25-µl PCR reaction in the presence of 1 U of Taq DNA polymerase (Fermentas, Glen Burnie, MD), 1x amplification buffer (with KCl), 0.1 µM each of the GeneRacer 5' primer and the GeneRacer 3' primer, 2.5 mM of $MgCl_4$, and 0.2 mM each of dNTPs. Amplifications were performed in a MyCycler thermal cycler (Bio-Rad) with the following cycling

temperatures: initial denaturation at 95 °C for 5 min, 20 cycles of denaturation for 30 s at 94 °C, annealing for 1 min at 68 °C and elongation for 3 min at 72 °C, and followed by a final extension for 15 min at 72 °C. Size of each PCR product was estimated by 1% agarose gel electrophoresis.

Direct sequencing of PCR products was performed using the Big Dye Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems, USA). The GeneRacer 5' primer was used to obtain the forward sequences. Automated sequencing was carried out using an ABI 3100 Genetic Analyzer (Applied Biosystems, Carlsbad, CA) and analyzed using the Applied Biosystems Sequencing software.

EST assembly and sequence analyses

All ABI-formatted chromatogram sequences were manually edited to remove poor-quality base reads and were converted to FASTA-formatted sequences by BioEdit (http://www.mbio.ncsu.edu/bioedit/bioedit.html). The FASTA sequences were assembled into contigs by the CAP3 program (http://deepc2.psi.iastate.edu/aat/cap/cap.html) [x01]. All unigenes were searched for significant similarities (E-value cut-off $\leq 1e^{-4}$) by the ESTexplorer, an online comprehensive ESTs management program that utilizes the BLASTX algorithm and the NCBI's non-redundant protein database (http://estexplorer.biolinfo.org) [x02, x03]. Annotated unigenes of *P. insidiosum* were searched against the previously-reported virulence genes of other pathogens in the literatures (i.e. Pubmed). The ESTexplorer also utilizes (i) ESTscan [x69] and InterProScan [x32] to predict protein domains, (ii) KOBAS [x70] to map proteins to metabolic pathways, and (iii) BLAST2GO [x71] to assign Gene Ontology (GO) term to each annotated unigene. All GO terms were categorized into groups by the CateGOrizer program [x06].

The open reading frame (ORFs) of each unigene was converted into an amino acid sequence using the Python programming language (http://www.python.org) and the Biopython [x49]. All deduced amino acid sequences were subjected to the SignalP 3.0 program (http://www.cbs.dtu.dk/services/SignalP) to predict signal peptides and cleavage sites [x04]. Using the criteria set by Win et al. [x05], a gene product was identified as an extracellular (secreted) protein, if the SignalP Hidden Markov Model (HMM) had a probability higher than 0.900, and the SignalP Neural Network (NN) predicted a cleavage site between amino acid position 10 and 40. To identify orthologs of the *P. insidiosum* genes in other oomycetes, BLASTX and TBLASTX analyses were performed against the genomes or ESTs of *P. ultimum* [x23], *P. sojae* [x22], *P. ramorum* [x22], *P. infestans* [x24], *H. arabidopsidis* [x84] and *S. parasitica*, at the VBI Microbial database (http://vmd.vbi.vt.edu/) and the Broad Institute database (http://www.broadinstitute.org/).

Phylogenetic analysis

Phylogenetic analyses were performed locally, using PHYLIP [x42a] and TreeView [x40], and online, using BioNJ [x42b] and TreeDyn [x43] via Phylogeny.fr [x41]. A total of 45 amino acid sequences of the elicitin domains from *P. insidiosum* [n=7; INSL1-7; this study] and from *Pythium oligandrum* [n=1], *Pythium vexans* [n=2], *Phytophthora cryptogea* [n=1], *Phytophthora brassicae* [n=3], *P. infestans* [n=9], *P. ramorum* [n=12] and *P. sojae* [n=10] (see Figure 5 for reference codes for obtaining protein sequences [x17]) were aligned by Clustal W program [x39]. Unrooted phylogenic trees of all elicitin domain sequences were generated by the Phylip and BioNJ programs using the neighbor-joining (NJ) algorithm.

Trees were viewed in the Treeview and TreeDyn programs. The reliability of the inferred trees was tested using bootstrap re-sampling with 1,000 replicates [x38].

Homology modeling of *P. insidisum* elicitins

Homology modeling for all elicitin-like (ELL) proteins of *P. insidiosum* (INSL1-7) was performed using the SWISS-MODEL (http://swissmodel.expasy.org/), according to the instructions described elsewhere [x53, x54, x55, x56]. Briefly, the elicitin (ELI) 1bxm chain A (the protein data bank (PDB) code: 1bxm) and 2pos chain D (the PDB code: 2pos) [x37, x52] were used as the prototypic templates to generate three-dimensional structures of INSL1-7. To predict whether *P. insidiosum* ELLs can bind ergosterol or cholesterol, the Dock Prep program (http://www.cgl.ucsf.edu/chimera/) [x57] was used to prepare the predicted three-dimensional structures of INSL1-7 for docking analysis. Then, the SwissDock (http://lausanne.isb-sib.ch/~agrosdid/projects/eadock/eadock_dss.php; http://swissdock.vital-it.ch/index.php) was used to analyze molecular docking between the *P. insidiosum* elicitins and the sterol ligands (the ZINC database IDs for cholesterol and ergosterol are 3875383 and 4084618, respectively (http://zinc.docking.org/) [x58]). Three-dimensional structures of ELI-sterol complexes were visualized by using the UCSF Chimera [x57].

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Generation of EST dataset: Full-length cDNAs, prepared from RNA extracted from young *P. insidiosum* mycelium grown at 37 °C, were cloned into the pCR4-blunt-TOPO vector and transformed into *E. coli* electrocompetent cells. A total of 486 clones were randomly selected for colony PCR using the GeneRacer 5' and 3' primers. Size of each PCR product (representing mRNA length) was evaluated by agarose gel electrophoresis. Of 486 ESTs obtained, 83% (n=403) were 400 – 900 bp long [Figure 1]. The shortest and the longest ESTs were ~200 and ~2200 bp, respectively. PCR products were sequenced using the GeneRacer 5' primer. Plasmid and adaptor sequences were manually removed using the BioEdit program. The CAP3 program [x01] assembled all ESTs to 217 unique sequences (so called "unigenes"): 137 singletons and 80 contigs. Each contig was derived from multiple clones, with 2 to 37 clones per unigene [Figure 1]. Of the 217 unigenes, 68% (n=147) appear to represent full-length transcripts, because they contained both the GeneRacer RNA oligo sequence (which is attached to the 5' end of full-length mRNAs) and poly-A tail.

Gene diversity of *P. insidiosum*: The *P. insidiosum* unigene sequences were annotated using ESTexplorer [x02, x03]. Although 73 unigenes (34%) did not match any protein in the database, 144 unigenes (66%) had significant hits (E-value < -4): 47 unigenes (22%) encoding ribosomal proteins and 97 unigenes (44%) encoding non-ribosomal proteins, including 9 hypothetical proteins [Table 1]. In addition, 13 of 73 unigenes with no significant BLASTX hits and 2 of 9 unigenes matched to a hypothetical proteins were assigned a protein domain by InterProScan [x32] [Table 2]. Thirty highly-representative unigenes (at least 4 clones per unigene) are shown in Table 3. Several sets of similar genes (gene families) were identified: 47 unigenes encode ribosomal proteins, 7 encode elicitin-like proteins (ELLs), 3 encode peptidylprolyl isomerases, and 2 each encode eukaryotic translation initiation factors, histones, RNA polymerases, small nuclear riboproteins, thioredoxin-like proteins, ubiquitins, and

V-type ATPase proteolipid subunits

The BLAST2GO program [x33] assigned 90 GO terms to 60 annotated unigenes. which can be divided into 3 categories: (i) Biological processes (37.8%), including proteins involved in cell organization and biogenesis, transport, nucleic acid binding, protein metabolism, developmental processes, cell cycle and proliferation, RNA metabolism, signal transduction and RNA transcription; (ii) Cellular components (28.9%), including proteins associated with specific sub-cellular fractions, including internal or plasma membrane, ribosome, mitochondrion, nucleus, cytosol, cytoskeleton, and vacuole; and (iii) Molecular function (33.3%), including transcription regulation, ribosome binding, kinase activity and transport. The KOBAS program [x34] indicated that 21 non-ribosomal protein-encoding genes are in 11 metabolic pathways: polyunsaturated fatty acid biosynthesis (PinsEST#104 and 146), methionine metabolism (PinsEST#037, 141 and 215), styrene degradation (PinsEST#103), tyrosine metabolism (PinsEST#074, 078 and 103), selenoamino acid metabolism (PinsEST#037, 141 and 215), chaperones and folding catalysts (PinsEST#067, 156, 158 and 200), protein folding and associated processing (PinsEST#016, 197 and 209), pyrimidine metabolism (PinsEST#021, 034 and 114), nitrogen metabolism (PinsEST#027 and 158), small cell lung cancer (PinsEXT#096), and C5-branched dibasic acid metabolism (PinsEST#069).

The SignalP 3.0 program [x04], with the criteria set by Win et al. [x05], predicts that 20 unigenes encode extracellular (secreted) proteins. Nine of them (PinsEST#007, 014, 026, 050, 072, 083, 084, 153 and 217) had no BLASTX hits, while the rest (n=11) include seven putative *P. insidiosum* ELLs (PinsEST#003, 025, 042, 045, 046, 066 and 176), two hypothetical proteins (PinsEST#149 and 151), and one each of an Os09g0542200-similar protein (PinsEST#052) and a cellulose binding elicitor lectin (CBEL; PinsEST#112).

Putative pathogenicity genes of *P. insidiosum*: Based on sequence homology to virulence genes of other pathogens, a handful of *P. insidiosum* genes encode putative virulence factors [Table 4]. These include ELLs, CBEL, and RXLR effector, which are pathogenicity proteins of plant-pathogenic oomycetes [x17, x47, x45]. Other *P. insidiosum* genes are predicted to encode enzymes with antioxidant functions, which can be important for pathogen survival in response to radical oxygen species produced by host organisms. These include superoxide dismutase [x10], thioredoxin-like protein [x15], and glutaredoxin [x11]. Predicted genes for calmodulin [x08] and heat shock transcription factor [x13] may facilitate growth and thermal adaptation at the animal host body temperature (37°C). There is a predicted *P. insidiosum* gene for β-carbonic anhydrase [x09]. This enzyme could promote a superficial infection on host surface (i.e., eye, skin) where CO_2 content is low. Finally, there are predicted genes with homology to macrophage migration inhibitory factor [x14] and ferrochelatase, an enzyme required for the final step in heme biosynthesis and is important for microbial virulence [x12].

Putative effectors of *P. insidiosum*: Effectors are a group of secreted molecules that manipulate host cell structure and function, in order to facilitate infection processes of many pathogens, including oomycetes [x68]. We identified *P. insidiosum* genes that encode effectors, including ELI or

ELL, CBEL, and RXLR homoloques. PinsEST#112 significantly matched the *P. parasitica* CBEL surface protein (E-value is 1.02E-09), and a "Conserved Domain Search" (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) revealed domains shared with the *P. parasitica* CBEL, including a signal peptide, and repeats of the PAN/Apple domain (four copies in PinsEST#112 vs. two in *P. parasitica*) [Figure 2]. In contrast, PinsEST#112 lacks the cellulose-binding domains of *P. parasitica* CBEL [Figure 2]. Since cell wall associated proteins are often O- and N-glycosylated, we used the NetOGlyc and NetNGlyc software [http://www.cbs.dtu.dk/services/NetNGlyc/] to predict O- and N-glycosylation sites and found two asparagine residuals (position 76 and 91) of PinsEST#112 protein with the predicted potential as N-glycosylation sites.

ELIs share the highly-conserved 98-amino acid elicitin domain with six cysteine residues, while ELLs contain either shorter or longer domains, with more diverse sequences [x17]. Seven ELLs were identified by BLASTX (n=6) and InterProScan (n=1) search, and were named INSL1 (PinsEST#003), INSL2 (PinsEST#025), INSL3 (PinsEST#042), INSL4 (PinsEST#045), INSL5 (PinsEST#046), INSL6 (PinsEST#066), and INSL7 (PinsEST#176). The mean protein length of all *P. insidiosum* ELLs, was 133 amino acids (range, 112 – 181), signal peptides were 16-20 amino acids long, the elicitin domain (Pfam PF00964) varied from 66 – 96 amino acids, and C-terminal domains were 5 – 51 amino acids long [Figure 2]. Only INSL2 and INSL7 had predicted O-glycosylation sites (20 and 13 sites, respectively). The big-PI Fungal Predictor [x36] identified no glycosylphosphatidylinositol (GPI) anchor in any *P. insidiosum* ELLs. The elicitin domain sequences of all *P. insidiosum* ELLs were aligned with that of some other oomycetes, such as, *Phytophthora cryptogea* (CRY; assession number, P15570.2), *Pythium vexans* (VEX1; assession number, AAB34416.1), *Phytophthora brassicae* (BRA2; assession number, AAO92424.1), *P. sojae* (SOJB; assession number, AAO24640.1), *P. ramorum* (RAM3B; assession number, ABB55984.1), and *P. infestans* (INF5; assession number, AAL16012.1) [Figure 4]. The alignment shows that all *P. insidiosum* ELLs have the six conserved cysteine residues, a main characteristic of elicitins (ELIs) for the predicted formation of 3 disulfide bonds [x49, x17] [Figure 4].

Structurally, an RXLR effector has an N-terminus portion that comprises a signal peptide (for secretion of effector), an RXLR-dEER domain (for translocation of effector), and a C-terminus functional domain, necessary for pathogenicity [x18, x19, x68]. The RXLR motif usually resides between the position 31 and 58, while that of the dEER motif is between positions 51 and 86 [x20, x21]. The dEER motif is less conserved than the RXLR motif, and characterized by a stretch of mainly acidic amino acids (D/E) [x18, x19]. To find a putative RXLR effector of *P. insidiosum*, the string "RXLR" was initially searched in all 217 predicted protein sequences. Proteins with this RXLR motif were then searched for the signal peptide and dEER motif. A total of 12 proteins contained the RXLR motif within the first 60 amino acids of the N-terminus. Two of these (PinsEST#107 and 113) had both RXLR and dEER motifs, but no predicted signal peptide. Only one protein (PinsEST#176) had a signal peptide and the RXLR-dEER domain. The gene products of PinsEST#107 and 113 had no BLAST hit, whereas that of PinsEST#176 was described above as an ELL (INSL7). The RXLR-dEER domains from INSL7 and four prototypic oomycete RXLR effectors (Avr1b from *P. sojae* [x79], Avr3a from *P. infestans* [x80], and ATR1 and ATR13 from *Hyaloperonospora parasitica* [x77, x78]) are aligned in Figure 3. All sequences have the conserved RXLR motif, but diverse dEER motif sequences. Similar to ATR13, the INSL7 has only one acidic amino acid (E) in the dEER motif.

Phylogenetic analysis of *P. insidiosum* elicitins: Jiang et. al. [x17] reported that 156 elicitin domain sequences from *Phytophthora* and *Pythium* species can be phylogenetically divided into 17 distinct clades: four ELIs and 13 ELLs. The elicitin domain sequences from all 7 ELLs (INSL1-7) of *P. insidiosum* and 38 ELIs/ELLs of other oomycetes [x17] were used to construct an unrooted phylogenetic tree based on Neighbor-Joining analysis with 1,000 bootstrap replicates. Analyses using the programs PHYLIP, TreeView, BioNJ and TreeDyn via Phylogeny.fr provided similar trees (Figure 5). Bootstrap scores $\geq$ 70% were present at each corresponding node. All of the elicitin domain sequences, except six from *P. insidiosum* (INSL1-6), fell into the 17 clades, as reported by Jiang et al. [x17]. Of all seven *P. insidiosum* ELLs, INSL7 was in clad ELL-06, and the rest, INSL1-6, were in novel clades: INSL1, INSL3 and INSL6 located in clade ELL-A, ELL-C and ELL-D, respectively, while INSL2, INSL4, and INSL5 formed clade ELL-B.

Predicted structure of *P. insidiosum* elicitins: Sequence alignment of 13 elicitin domain sequences, including that of the structure- and function-defined elicitin CRY [x37], showed up to 16 possible ergosterol interactive sites in the elicitin domains of *P. insidiosum* ELLs [Figure 4]. Using homology modeling based on the structure-defined elicitin 1bxm [x37] and 2pos [x52], all *P. insidiosum* elicitins, except INSL7, have predicted three-dimentional structures with a small pocket-like cavity. Protein-ligand docking analysis using SwissDock (see the methods for details) predicts that three *P. insidiosum* elicitins (INSL1, INSL2 and INSL5) could encapsulate either cholesterol or ergosterol in the cavity. Predicted structures of the complex between INSL2 and cholesterol are depicted in Figure 6.

EST comparative analysis: BLASTX and TBLASTX were used to search protein databases from the genomes of six oomycete species: *P. ultimum*, *P. sojae*, *P. ramorum*, *P. infestans*, *H. arabidopsidis* and *S. parasitica*. These species were chosen because of the availability of their genome sequences. While most of our ESTs showed homology to other sequences in these oomycete databases, we found one putative gene, with unknown function, that is unique to *P. insidiosum* (PinsEST#086). Orthologs of PinsEST#119 are only found in *P. infestans* (AAY43427.1) and *S. parasitica* (SPRG_10907) genomes. Sequence alignment and domain analyses showed that PinsEST#119 and its *S. parasitica* orthologs contain a domain of the *Rickettsia* 17-kDa surface antigen, while that of *P. infestans* does not (data not shown).

Note: Please see the tables, figures, and references in the full manuscript in the appendix

โครงการย่อยที่ 3A: Development of a rapid serological test for diagnosis of human pythiosis

วัตถุประสงค์: เพื่อพัฒนาชุดตรวจเลือด สำหรับช่วยวินิจฉัยโรค human pythiosis ที่รวดเร็ว มีประสิทธิภาพและสามารถนำไปใช้ในพื้นที่ห่างไกลได้: immunochromatography

วิธีการวิจัย

Microorgansim and growth condition: The *Pythium insidiosum* strain CBS119452 isolated from Thai patients with vascular pythiosis was used to prepare antigen in this study. The organism had been maintained on Sabouruad dextrose agar at 37°C until antigen preparation.

Antigen preparation: The *P. insidiosum* CBS119452 isolate was subcultured on Sabouraud dextrose agar and incubated at 37°C for 2 days. Several small agar pieces containing hyphal elements from the growing culture were transferred to 200 ml of Sabouraud dextrose broth and shaken (150 rpm) at 37°C for one week. Merthiolate (final concentration 0.02% wt/v) was added to kill the cultures before they were filtered through a Durapore membrane filter (0.22-μm pore size; Millipore, County Cork, Ireland). PMSF (0.1 mg/ml) and EDTA (0.3 mg/ml) were added to minimize protein degradation in the filtrated broth before it was concentrated ~80 folds using an Amicon Ultra-15 centrifugal filter (10,000 nominal molecular weight limit (NMWL); Millipore, Bedford, MA). The concentrated filtered broth was referred to as culture filtrate antigen (CFA), and was measured for protein concentration by spectrophotometer. The CFA was stored at 4°C until use.

Serum samples: A total of 33 sera from known cases of human pythiosis (27 vascular, 4 ocular, and 2 cutaneous) were recruited for test evaluation. Diagnosis of human pythiosis was based on previously reported criteria (9): (i) culture isolation of *P. insidiosum* (n=15), (ii) serodiagnosis (n=9), and (iii) presence of the unique clinicopathological features of vascular pythiosis (n=9). Additional 181 sera were collected for use in 4 control groups. The first group included 100 sera randomly collected from healthy blood donors who came to the Blood Bank Division, Ramathibodi Hospital. The second group included 19 healthy thalassemic patients with no clinical evidence for pythiosis. The third group included sera from 6 patients with non-infectious diseases (5 highly-positive antinuclear antibody titer and 1 thromboangiitis obliteran (TAO)). The forth group included 56 sera from patients with other infections (7 each of penicillosis and galactomannan positive, 6 cryptococcosis, 5 malaria, 4 each of aspergillosis and mycoplasmosis, 3 each of zygomycosis, histoplasmosis, syphilis, and anti-human immunodeficiency virus positive, 2 each of toxoplasmosis, leptospirosis, and mellioidosis, and 1 each of amoebiasis, disseminated candidiasis, anti-hepatitis A virus positive, anti-hepatitis B virus positive, and anti-hepatitis C virus positive). All sera were kept at -20°C until use.

Immunochromatographic test:

Conjugation of antibody to colloidal gold: The 40-nm colloidal gold suspension (Arista, Allentown, PA) was adjusted to pH 9.65 by using 0.2 M Na_2CO_3. To each 500 μl of colloidal gold, 3 μg rabbit anti-human IgG (Dako, Glostrup, Denmark) was added and incubated for 30 min at room temperature. The residual surface of the colloidal gold particles was blocked by 5% (wt/v) bovine serum albumin (BSA; Sigma, St. Louis, Mo) and incubated for 10 min. The conjugate was centrifuged at 6,000g for 15 min and the supernatant was then discarded. The conjugate pellet was washed in 0.5% (wt/v) casein and

centrifuged at 6,000g for 15 min. After removing the supernatant, the conjugate was pooled and resuspended in 0.5% (wt/v) casein and 20% (wt/v) sucrose in 0.02 M Tris-HCl (pH 8.0) with 40-time less volume than the original. This IgG–colloidal gold conjugate (2.5μl) was impregnated on a piece of 2.5x2.5mm glass fiber (GF33; Whatman/Schleicher & Schuell, Dassel, Germany) and dried in a dehumidifier cabinet for an hour.

Immobilization of antigen and antibody onto nitrocellulose membrane: A 1.5cm-width nitrocellulose membrane (AE99; Whatman/Schleicher & Schuell, Dassel, Germany) was lined with CFA (1:5 dilution; the test line) and sheep anti-rabbit IgG (150 μg/ml in 50 mM ammonium acetate buffer, pH 4.5; the control line) at 1 μl/cm, by a dispenser (Biodot ZX 1000, Bio-Dot, Irvine, CA) (Figure 1A, B). The membrane was dried, blocked with 1% (wt/v) BSA, and dried again in a dehumidifier cabinet.

Assembly of ICT strip: The immobilized nitrocellulose membrane, glass fiber with the colloidal gold conjugate, sample pad (903 paper; Whatman/Schleicher & Schuell, Dassel, Germany) and wicking pad (3MM chromatography; Whatman, Maidstone, England) were assembled on a backing plastic, and was then cut into 2.5mm-width strips by the strip-cutting machine (Biodot CM 4000 R, Bio-Dot, Irvine, CA) (Figure 1A, B).

Detection of human anti-P. insidiosum antibody by ICT: Each individual sample was diluted to 1:10,000 in phosphate buffer (pH 7.4). The ICT was tested in duplicate in 100 μl of diluted sera in 96-well microtiter plate. Test signal of each ICT strip was read by naked eye at 30 min by 3 independent laboratory personnels. To quantify ICT signal, each strip was scanned by a scanner (Epson perfection 1670 photo, Seiko Epson Crop., Japan) to obtain tagged image file format picture. Test and background signal intensities were measured by the Quantity-One program (Bio-Rad). Intensity value derived from a test signal subtracted by a background signal was referred as ICT value (IV). Sensitivities and specificities were calculated for all cutoff levels of IV and graphically displayed in receiver-operating characteristic (ROC) curves using the Stata v10 program (StataCorp, Texus, USA).

Immunodiffusion test: The ID test was modified from the method of Pracharktam et al. (18). Briefly, agar gel diffusion was carried out on a 5-cm-diameter Petri dish with 2% agar in Veronal buffer (0.9% (wt/v) $C_8H_{11}N_2NaO_3$, 0.05% (wt/v) NaN_3, pH 8.6). The CFA and serum to be tested were each added to 4-mm-diameter wells separated by 4 mm. The Petri dish was incubated in a moist chamber at room temperature for 24 hr. The appearance of a precipitation line by the eye was considered a positive test result.

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Development of ICT: The components of an ICT strip are depicted in Figure 1. CFA was blotted on nitrocellulose membrane (indicated as test line), and used as the specific *P. insidiosum* antigen for detecting anti-*P. insidiosum* IgG in serum samples. Sheep anti-rabbit IgG (indicated as control line) was blotted distal to CFA. When human IgGs in serum moved upward by capillary action through the glass fiber, they formed complexes with the rabbit anti-human IgG-colloidal gold conjugate. The complexes migrated through the nitrocellulose membrane. Immune complexes containing human anti-*P. insidiosum* IgG bound CFA and developed a purple signal at the test line. In contrast, immune complex lacking human anti-*P. insidiosum* IgG passed through the test line without developing a signal.

The sheep anti-rabbit IgG bound the remaining immune complexes containing rabbit anti-human IgG-colloidal gold conjugate, and exhibited an internal test validation signal at the control line.

Diagnostic performance of ICT in comparison to ID: ICT and ID results were read by 3 independent laboratory personnels. Based on results from all sera of pythiosis patients (27 vascular, 4 ocular, and 2 cutaneous) and the control groups, ICT showed 88% sensitivity, 100% specificity, 100% positive predictive value (PPV), and 98% negative predictive value (NPV), while ID showed 61% sensitivity, 100% specificity, 100% PPV, and 93% NPV, respectively. False negative ICT results were obtained from sera of all ocular pythiosis patients. False negative ID results were obtained from sera of all ocular pythiosis patients, 8 patients with vascular pythiosis, and 1 patient with cutaneous pythiosis.
ICT signals generated from all sera were quantified and converted to IV unit (see the method; Figure 2). Mean IV of vascular pythiosis patients was 67.2 units (Range, 22.4-113.3), whereas that of cutaneous pythiosis patients was 17.7 (16.0-19.4), ocular pythiosis patients was 4.3 (0-7.4), blood donors was 3.8 (0-12.3), patients with other infectious diseases was 2.5 (0-11.9), thalassemic patients was 2.7 (0-5.9), and patients with autoimmune diseases and TAO was 2.1 (0-3.8). Alternative to determining the result by 3 independent laboratory personnels, the IV cutoff point was selected by a ROC analysis to differentiate between patients with or without human pythiosis. ICT has a very good discriminative power for identifying patients with human pythiosis as shown by the large area under the curve (0.95) of the ROC curve (Figure 3). The IV of 16.0 was selected as the cutoff value, because it gave the highest sum of sensitivity (88%) and specificity (100%). For comparison, the IV cutoff value of 12.3 yielded a sensitivity of 88% and specificity of 99%, and the IV cutoff value of 19.4 yielded a sensitivity of 85% and a specificity of 100%.

Note: Please see the tables, figures, and references in the published version in the appendix

โครงการย่อยที่ 3B: Development of a rapid serological test for diagnosis of human pythiosis

วัตถุประสงค์: เพื่อพัฒนาชุดตรวจเลือด สำหรับช่วยวินิจฉัยโรค human pythiosis ที่รวดเร็ว มีประสิทธิภาพและสามารถนำไปใช้ในพื้นที่ห่างไกลได้: Hemagglutination test

วิธีการวิจัย

Serum samples: A total of 33 sera from patients with pythiosis (27 vascular, 4 ocular, and 2 cutaneous) were recruited for the assay evaluation. Clinical information was recorded for each pythiosis patient and included clinical features, duration of symptoms before the first medical visit, underlying diseases and method of diagnosis (Table 1). All pythiosis patients were diagnosed based on at least one of following criteria: (i) *P. insidiosum* isolated from infected tissue and confirmed by induction and identification of zoospores, or (ii) presence of anti–*P. insidiosum* antibodies in blood samples, as detected by at least one of the following well-established serodiagnostic tests: ID test, ELISA, Western blot analysis, or ICT (3, 11-13, 15-18, 20-23, 25, 32). Additional sera (n=289) were collected as control samples that included (i) 186 randomly-collected sera from healthy blood donors at the Blood Bank Division of Ramathibodi Hospital; (ii) 21 sera from healthy thalassemic patients without clinical evidence of pythiosis; (iii) 5 sera from patients with highly positive antinuclear antibody; and (iv) 77 sera from patients positive for other infectious diseases. The latter included 19 sera obtained from patients with proven cryptococcosis (n=11), penicillosis (n=7) or candidiasis (n=1) as determined by criteria for invasive fungal diseases of the European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group (EORTC/MSG)(6). Of the remaining 58 sera, 20 were obtained from patients with aspergillosis (n=4) or mucormycosis (n=4) confirmed by culture identification, from patients (n=9) that were fungal galactomannan antigen positive, and from patients (n=3) that were anti-*Histoplasma capsulatum* antibody positive. However, information on host factors, clinical features and other mycological evidence for revalidation by EORTC/MSG criteria were missing for these 20 sera. The remaining 38 out of 77 sera comprised samples from cases with proven non-fungal infections according established criteria (4, 34). These included samples that were positive for anti-human immunodeficiency virus (n=10), syphilis (n=9), malaria (n=5), mycoplasmosis (n=4), toxoplasmosis (n=2), leptospirosis (n=2), melliodosis (n=2), anti-*Entamoea histolytica* antibody (n=1), hepatitis A virus (n=1), hepatitis B virus (n=1), and hepatitis C virus (n=1). One positive-control serum sample was obtained from a rabbit immunized with *P. insidiosum* antigen. All sera were stored at -20°C until used.

Antigen preparation: The *P. insidiosum* strain CBS119452 isolated from a Thai patient with vascular pythiosis was used to prepare antigen. The microorganism was subcultured on Sabouraud dextrose agar and incubated at 37°C for 3 days. Several small blocks of mycelium were transferred to Sabouraud dextrose broth and shaken (150 rpm) at 37°C for 9 days. Merthiolate was added to the culture at a final concentration of 0.02% (wt/vol). The culture was filtered through a Durapore membrane filter (0.22-μm pore size). Phenylmethylsulfonyl fluoride (0.1mg/ml) and EDTA (0.3 mg/ml) were added to the culture filtrate broth before concentration to ~80-fold using an Amicron® 8400 apparatus and an Amicon Ultra-15 centrifugal filter (10,000 nominal molecular weight limit; Millipore, Bedford, MA). The

concentrated broth was referred to as culture filtrate antigen (CFA), and was measured for protein concentration by spectrophotometer. CFA was stored at -20°C.

Hemagglutination test:

Stabilization of sheep red blood cells: Preparation of sheep red cells (SRC) for the HA test was modified from the methods of Hirata et. al. and Petchclai et. al. (7, 24). The SRC were packed by washing with normal saline and centrifuging at 5,000 rpm for 3 minutes, for 3 times. Then, 1.25 ml of 0.15 M phosphate-buffered saline (pH 7.2) (PBS) and 0.25 ml of 2.5% glutaraldehyde in distilled water were added to 0.1 ml of the packed SRC followed by gentle mixing by rotator at room temperature for 2 h. The SRC were washed with normal saline and centrifuged 3 times as above. PBS with 0.1% sodium azide was added to the SRC to make a 10% glutaraldehyde-stabilized SRC suspension.

Preparation of P. insidiosum antigen-coated SRC: To coat SRC with *P. insidiosum* antigens, 0.1 ml of 2 mg/ml CFA and 1 ml of 0.1 M acetate buffer (pH 4) were sequentially added to 0.1 ml of 10% glutaraldehyde-stabilized SRC suspension, before mixing and incubating at 37^{O}C for 30 min. The CFA-coated SRC were washed with normal saline and centrifuged 3 times (as above), and re-suspended in PBS with 0.1% bovine serum albumin and 0.1% sodium azide to make a 0.5% CFA-coated SRC suspension.

Hemagglutination assay: To perform the HA test, 25 µl of 1:10-diluted serum was added to the first well of a 96 U-shaped-well microtiter plate. Then, the serum was diluted 2-fold from 1:10 to 1:20,480 using diluent (0.5% bovine serum albumin, 1% normal rabbit serum and 0.1% sodium azide in PBS). Twenty-five µl of the 0.5% CFA-coated SRC suspension was added to each well and mixed gently. The positive control well contained the CFA-coated SRC suspension mixed with a positive serum. The negative control wells contained CFA-coated SRC suspension and the diluent or a negative serum. Each sample was tested in duplicate. The plate was incubated for 1 h at room temperature. The presence of hemagglutination was read as a positive test result for *P. insidiosum*, whereas the absence of hemagglutination was read as a negative test result.

Immunodiffusion test: The ID test was modified from the original method of Pracharktam et. al. (25). Briefly, agar gel diffusion was carried out in a 5-cm-diameter Petri dish with 2% agar in Veronal buffer (0.9% (wt/v) $C_8H_{11}N_2NaO_3$, 0.05% (wt/v) NaN_3, pH 8.6). The CFA and a serum were each added to 4-mm-diameter wells that were set 4 mm apart from each other. The plates were incubated in a moist chamber at room temperature for 24 h. The presence of an identity precipitation line with the positive control serum was considered as a positive test result for *P. insidiosum*.

Statistical analysis: Sensitivities, specificities and accuracies were calculated from each cutoff titer of the HA test and displayed in a receiver-operating characteristic (ROC) curve using the Stata program Version 10.0 (StataCorp, Texus, USA).

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The area under the ROC curve for the HA test results was 0.99 (Figure 1). The best cutoff titer chosen for the HA test was 1:160 because it provided the highest accuracy (98.1%; Table 2). At this cutoff titer, all 29 sera from vascular and cutaneous pythiosis patients, serum from one healthy blood donor and serum from a patient with mycoplasmosis (i.e., 2 from the negative control group) gave positive test results, while all 4 sera from the ocular pythiosis patients and the remaining 287 sera from

the negative control patients gave negative test results. Therefore, sensitivity and specificity of the HA test were 87.9% and 99.3%, respectively. Incubation time for the HA test was 1 h.

To calculate positive and negative predictive values of the HA test, prevalence of pythiosis, test sensitivity (88%), and test specificity (99%) were used as in the statistic equation described elsewhere (1). At Ramathibodi hospital during year 2008, the prevalence of pythiosis in all patients (n=190,415) and in patients (n=372) with thalassemia (a prominent predisposing factor for pythiosis) were 0.002% and 0.8%, respectively. The PPV and NPV of the HA test using the prevalence in all patients were 0.2% and 100.0%, respectively, while the PPV and NPV using the prevalence in thalassemic patients were 41.7% and 99.9%, respectively.

To test the precision of the HA test, one each of the control positive and negative control sera were repeatedly assayed 20 times in both within-run and between-run analyses. Highest HA titers obtained from each precision analysis were 1:640 for the positive control serum and negative at all titers for the negative control serum. The CFA-coated SRC were stable for at least 6 months since reactions of the positive control serum remained unchanged at the highest HA titer (1:640) over the interval (i.e., at 2 wks, 1 mo, 3 mo, 5 mo and 6 mo after the preparation of coated-SRC).

For the ID test, only 20 sera from patients with vascular (n=19) and cutaneous (n=1) pythiosis gave positive results. All of the control sera and 13 sera from patients with vascular (n=8), ocular (n=4) and cutaneous (n=1) pythiosis gave negative test results. Therefore, sensitivity, specificity and accuracy of the ID test were 60.6%, 100.0% and 96.0%, respectively. Incubation time for ID test was ~24 h.

Note: Please see the tables, figures, and references in the published version in the appendix

OUTPUT ที่ได้จากโครงการวิจัย

PUBLICATIONS: [* = corresponding author]

1. **Krajaejun T** *, Imkhieo S, Intaramat A, Ratanabanangkoon K*. Development of an Immunochromatographic Test for Rapid Serodiagnosis of Human Pythiosis. Clin Vaccine Immunol 2009; 16: 506-9

2. Jindayok T, Piromsontikorn S, Srimuang S, Khupulsup K, **Krajaejun T** *. Hemagglutination Test for Rapid Serodiagnosis of Human Pythiosis. Clin Vaccine Immunol 2009; 16: 1047-51.

3. **Krajaejun T** *, Keeratijarut A, Sriwanichrak K, Lowhnoo T, Rujirawat T, Petchthong T, Yingyong W, Kalambaheti T, Smittipat N, Juthayothin T, Sullivan TD. The 74-kiloDalton Immunodominant Antigen of the Pathogenic Oomycete Pythium insidiosum is a Putative Exo-1,3-ß Glucanase. Clin Vaccine Immunol 2010; 17:1203-10.

4. **Krajaejun T** *, Khositnithikul R, Lerksuthirat T, Lowhnoo T, Rujirawat T, Petchthong T, Yingyong W, Suriyaphol P, Smittipat N, Juthayothin T, Phuntumart V, Sullivan TD. Expressed Sequence Tags Reveal Genetic Diversity and Putative Virulence Factors of the Pathogenic Oomycete *Pythium Insidiosum* (manuscript is grammar edited, and about to submit to a journal)

PATENT PENDING:

1. Imkhieo S, Intaramat A, **Krajaejun T**, Ratanabanangkoon K, inventors; Chulabhorn Research Institute; Mahidol University, assignee. [Development of a simple immunochromatographic test for diagnosis of pythiosis]. Thailand patent application no. 0801004950. 2008 Sep 25. Thai.

2. Jindayok T, Piromsontikorn S, Srimuang S, Khupulsup K, **Krajaejun T,** inventors; Mahidol University, assignee. [A hemagglutination test for rapid serodiagnosis of human pythiosis]. Thailand patent application no. 1003000276. 2010 Mar 26. Thai.

ภาคผนวก

A. Manuscript: โครงการวิจัยย่อยที่ 2

B. Reprint: โครงการวิจัยย่อยที่ 1

C. Reprint: โครงการวิจัยย่อยที่ 3A

D. Reprint: โครงการวิจัยย่อยที่ 3B

E. บทความเผยแพร่:

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กรุงเทพธุรกิจ

RAMA plus magazine

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TITLE: Expressed Sequence Tags Reveal Genetic Diversity and Putative Virulence Factors of the Pathogenic Oomycete *Pythium insidiosum*

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ABSTRACT

Oomycetes are unique eukaryotic microorganisms that share a mycelial morphology with fungi. Many oomycetes are pathogenic to plants, while some oomycetes are pathogenic to animals. *P. insidiosum* is the only oomycete that is capable of infecting both humans and animals, and causes a life-threatening infectious disease, called "pythiosis". In the majority of pythiosis patients life-long handicaps result from the inevitable radical excision of infected organs, and many die from advanced infection. Better understanding *P. insidiosum* pathogenesis at molecular levels could lead to new forms of treatment. Genetic and genomic information is lacking for *P. insidiosum*, so we have undertaken an EST study, and report on the first dataset of 486 ESTs, assembled into 217 unigenes. Of these, 144 had significant sequence similarity with know genes, including 47 with ribosomal protein homology. Potential virulence factors included genes involved in antioxidation, thermal adaptation, immunomodulation, and iron and sterol binding. Effectors resembling pathogenicity factors of plant-pathogenic oomycetes, were also discovered, such as, a CBEL-like protein (possible involvement in host cell adhesion and hemagglutination), a putative RXLR effector (possibly involved in host cell modulation) and elicitin-like (ELL) proteins. Phylogenetic analysis mapped *P. insidiosum* ELLs to several novel clades of oomycete elicitins, and homology modeling predicted that *P. insidiosum* elicitins should bind sterols. Most of the *P. insidiosum* ESTs showed homology to sequences in the genome or ESTs databases of other oomycetes, but one putative gene, with unknown function, was found to be unique to *P. insidiosum*. The EST dataset reported here represents the first steps in identifying genes of *P. insidiosum* and beginning transcriptome analysis. This genetic information will facilitate understanding of pathogenic mechanisms of this devastating pathogen.

INTRODUCTION

Oomycetes are eukaryotic organisms that belong to the Stramenopiles of the supergroup Chromalveolates, and comprise many genera, including *Pythium*, *Albugo*, *Peronospora*, *Phytophthora*, *Plasmopara*, *Bremia*, *Aphanomyces*, *Lagenidium* and *Saprolegnia* [x59, x60]. These organisms share microscopic morphology with the fungi. However, phylogenetic analysis demonstrates that the oomycetes are more closely related to diatoms and algae than the true fungi [x61]. Many oomycetes are pathogenic to plants, while some oomycetes (e.g. *Pythium* spp., *Aphanomyces* spp., *Lagenidium* spp., and *Saprolegnia*

spp.) are pathogenic to humans and animals, such as, horses, dogs, cats, cattle, fish, and mosquitoes [x59]. Since anti-mycotic agents are mostly ineffective in controlling the oomycetes, treating infection caused by the oomycetes is problematic and challenging.

To date, *P. insidiosum* appears to be the only oomycete that is capable of infecting both humans and animals, causing a life-threatening infectious disease, called pythiosis. The most common sites of infection in humans are the arteries and eyes [x62], while in animals cutaneous/subcutaneous and gastrointestinal infections dominate [x63]. Probably because the pathogen is sensitive to low temperature [x65], pythiosis is more prevalent in tropical and subtropical areas of the world [x63]. Natural habitats of *P. insidiosum* are swampy areas, where the organism is present as broad, right-angle branching mycelium. Like other oomycetes, *P. insidiosum* produce motile, biflagellate asexual zoospores, which germinate to produce mycelia on natural hosts (water plants) [x64]. The zoospore, considered the infective morphotype, also adheres to surface of humans or animals, and germinates as hyphae into host tissues [x64].

High rates of morbidity and mortality of pythiosis are exacerbated by the lack of effective treatment [x62, x63]. Available antifungal drugs have been tested for treatment of pythiosis, but have been mostly unsuccessful. This failure likely results from the lack of the drug-targeted ergosterol biosynthesis pathway [x62, x66]. Radical excision of infected tissues or organs (i.e., eyes, legs) is the main option for treatment of pythiosis [x62, x63]. The extensive surgeries result in life-long handicaps for the majority of pythiosis patients, and many die from an advanced and uncontrolled infection. An immunotherapeutic vaccine, prepared from *P. insidiosum* crude proteins, showed limited success, with rates of disease clearance of ~50% for humans, ~30% for dogs, and ~60% for horses with pythiosis [x62, x67]. Thus, improvement of final outcomes for effective treatment of patients, who suffer from pythiosis, is still an important healthcare goal.

Little is known about biology and pathogenesis of *P. insidiosum* at the molecular level. Only four genes of *P. insdisiosum* have been reported in GenBank. These include the rRNA gene cluster, as well as genes for exo-1,3-beta glucanase, chitine synthase 2, and cytochrome c oxidase subunit II. The genome sequence of *P. insidiosum*, a crucial resource for exploring the pathogen, is not available, although those of several other plant- and animal-pathogenic oomycetes are published, including *Phytophthora sojae*, *Phytophthora ramorum*, *Phytophthora infestans*, *Phytophthora capsici*, *Hyaloperonospora arabidopsidis*, *Pythium ultimum*, and *Saprolegnia parasitica* [x22, x23, x24, x25, x84]. As an alternative to the generation of a whole genome sequence, which is expensive and time consuming, we have undertaken the faster and less costly generation of expressed sequence tags (EST) of *P. insodiosum*, as an effective way to discover new genes and to explore genetic diversity between organisms within the oomycetes and with more distantly related species [x26, x27, x28, x29, x30, x31]. We report the first EST dataset for *P. insodiosum*, provide initial assessments of gene expression for *P. insidiosum*, and through sequence comparison with other *oomycetous* species, identify possible targets for the discovery of novel strategies for the treatment of pythiosis.

MATERIALS AND METHODS

Strain, growth condition and RNA extraction

The *P. insidiosum* strain CBS119452, isolated from a Thai patient with vascular pythiosis, was used to prepare RNA. The microorganism was cultured in a Petri dish containing 10 ml of Sabouraud dextrose broth and incubated at 37 C for 5 days. Two loops full of growing mycelium were disrupted with glass beads (diameter, 710-1,180 µm; Sigma, St. Louis, MO), using TissueLyzer MM301 (Qiagen, Germany). Total RNA was extracted using Trizol Reagents (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. The RNA concentration and purity were measured using spectrophotometry (UV-1700, Shimadzu Corp., Japan), and the integrity was evaluated by agarose gel electrophoresis. The RNA was stored at -80 °C until use.

Construction of cDNA library

Full-length cDNAs of *P. insidiosum* were generated from 3.5 µg of total RNA, using the GeneRacer kit (Invitrogen) according to the manufacturer's protocol. Using 0.5 µl of freshly-prepared cDNAs as template, they were amplified with 0.025 U of proof-reading Platinum *Pfx* DNA polymerase (Invitrogen), in 1x *Pfx* amplification buffer with 1 mM of $MgSO_4$, 0.3 mM each of dNTPs, 0.9 µM each of the GeneRacer 5' primer (5′-CGACTGGAGCACGAGGACACTGA-3′) and the GeneRacer 3' primer (5′-GCTGTCAACGATACGCTACGTAACG-3′), in a MyCycler thermal cycler (Bio-Rad, Hercules, CA) with the following cycling temperatures: an initial denaturation at 94 °C for 2 min, and a total of 35 amplification cycles: 5 cycles of denaturation for 30 s at 94 °C and annealing and elongation for 3 min at 72 °C, 5 cycles of denaturation for 30 s at 94 °C and annealing and elongation for 3 min at 70 °C, 25 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at 60 °C and elongation for 3 min at 68 °C, and followed by a final extension for 10 min at 68°C. The PCR products were assessed for the expected heterogeneous-sized bands by 1% agarose gel electrophoresis. The PCR products were inserted to the pCR4-blunt-TOPO vector using the Zero Blunt TOPO PCR cloning kit (Invitrogen) and then transformed to the electrocompetent *Escherichia coli* strain DH5α, according to the manufacturer's protocol. The

transformed bacteria were incubated overnight on LB agar containing 100 µg of ampicillin.

Colony PCR and DNA sequencing

A small portion of a transformed bacterial colony was suspended in 5 µl sterile water (to serve as PCR template) and was all added to a 25-µl PCR reaction in the presence of 1 U of Taq DNA polymerase (Fermentas, Glen Burnie, MD), 1x amplification buffer (with KCl), 0.1 µM each of the GeneRacer 5' primer and the GeneRacer 3' primer, 2.5 mM of $MgCl_4$, and 0.2 mM each of dNTPs. Amplifications were performed in a MyCycler thermal cycler (Bio-Rad) with the following cycling temperatures: initial denaturation at 95 °C for 5 min, 20 cycles of denaturation for 30 s at 94 °C, annealing for 1 min at 68 °C and elongation for 3 min at 72 °C, and followed by a final extension for 15 min at 72 °C. Size of each PCR product was estimated by 1% agarose gel electrophoresis.

Direct sequencing of PCR products was performed using the Big Dye Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems, USA). The GeneRacer 5' primer was used to obtain the forward sequences. Automated sequencing was carried out using an ABI 3100 Genetic Analyzer (Applied Biosystems, Carlsbad, CA) and analyzed using the Applied Biosystems Sequencing software.

EST assembly and sequence analyses

All ABI-formatted chromatogram sequences were manually edited to remove poor-quality base reads and were converted to FASTA-formatted sequences by BioEdit (http://www.mbio.ncsu.edu/bioedit/bioedit.html). The FASTA sequences were assembled into contigs by the CAP3 program (http://deepc2.psi.iastate.edu/aat/cap/cap.html) [x01]. All unigenes were searched for significant similarities (E-value cut-off $\leq 1e^{-4}$) by the ESTexplorer, an online comprehensive ESTs management program that utilizes the BLASTX algorithm and the NCBI's non-redundant protein database (http://estexplorer.biolinfo.org) [x02, x03]. Annotated unigenes of *P. insidiosum* were searched against the previously-reported virulence genes of other pathogens in the literatures (i.e. Pubmed). The ESTexplorer also utilizes (i) ESTscan [x69] and InterProScan [x32] to predict protein domains, (ii) KOBAS [x70] to map proteins to metabolic pathways, and (iii) BLAST2GO [x71] to assign Gene Ontology (GO) term to each annotated unigene. All GO terms were categorized into groups by the CateGOrizer program [x06].

The open reading frame (ORFs) of each unigene was converted into an amino acid sequence using the Python programming language (http://www.python.org) and the Biopython [x49]. All deduced amino acid sequences were subjected to the SignalP 3.0 program (http://www.cbs.dtu.dk/services/SignalP) to predict signal peptides and cleavage sites [x04]. Using the criteria set by Win et al. [x05], a gene product was identified as an extracellular (secreted) protein, if the SignalP Hidden Markov Model (HMM) had a probability higher than 0.900, and the SignalP Neural Network (NN) predicted a cleavage site between amino acid position 10 and 40. To identify orthologs of the *P. insidiosum* genes in other oomycetes, BLASTX and TBLASTX analyses were performed against the genomes or ESTs of *P. ultimum* [x23], *P. sojae* [x22], *P. ramorum* [x22], *P. infestans* [x24], *H. arabidopsidis* [x84] and *S. parasitica*, at the VBI Microbial database (http://vmd.vbi.vt.edu/) and the Broad Institute database (http://www.broadinstitute.org/).

Phylogenetic analysis

Phylogenetic analyses were performed locally, using PHYLIP [x42a] and TreeView [x40], and online, using BioNJ [x42b] and TreeDyn [x43] via Phylogeny.fr [x41]. A total of 45 amino acid sequences of the elicitin domains from *P. insidiosum* [n=7; INSL1-7; this study] and from *Pythium oligandrum* [n=1], *Pythium vexans* [n=2], *Phytophthora cryptogea* [n=1], *Phytophthora brassicae* [n=3], *P. infestans* [n=9], *P. ramorum* [n=12] and *P. sojae* [n=10] (see Figure 5 for reference codes for obtaining protein sequences [x17]) were aligned by Clustal W program [x39]. Unrooted phylogenic trees of all elicitin domain sequences were generated by the Phylip and BioNJ programs using the neighbor-joining (NJ) algorithm. Trees were viewed in the Treeview and TreeDyn programs. The reliability of the inferred trees was tested using bootstrap re-sampling with 1,000 replicates [x38].

Homology modeling of *P. insidisum* elicitins

Homology modeling for all elicitin-like (ELL) proteins of *P. insidiosum* (INSL1-7) was performed using the SWISS-MODEL (http://swissmodel.expasy.org/), according to the instructions described elsewhere [x53, x54, x55, x56]. Briefly, the elicitin (ELI) 1bxm chain A (the protein data bank (PDB) code: 1bxm) and 2pos chain D (the PDB code: 2pos) [x37, x52] were used as the prototypic templates to generate three-dimensional structures of INSL1-7. To predict whether *P. insidiosum* ELLs can bind ergosterol or cholesterol, the Dock Prep program (http://www.cgl.ucsf.edu/chimera/) [x57] was used to prepare the predicted three-dimensional structures of INSL1-7 for docking analysis. Then, the SwissDock (http://lausanne.isb-sib.ch/~agrosdid/projects/eadock/eadock_dss.php; http://swissdock.vital-it.ch/index.php) was used to analyze molecular docking between the *P. insidiosum* elicitins and the sterol ligands (the ZINC database IDs for cholesterol and ergosterol are 3875383 and 4084618, respectively

(http://zinc.docking.org/) [x58]). Three-dimensional structures of ELI-sterol complexes were visualized by using the UCSF Chimera [x57].

RESULTS

Generation of EST dataset

Full-length cDNAs, prepared from RNA extracted from young *P. insidiosum* mycelium grown at 37 °C, were cloned into the pCR4-blunt-TOPO vector and transformed into *E. coli* electrocompetent cells. A total of 486 clones were randomly selected for colony PCR using the GeneRacer 5' and 3' primers. Size of each PCR product (representing mRNA length) was evaluated by agarose gel electrophoresis. Of 486 ESTs obtained, 83% (n=403) were 400 – 900 bp long [Figure 1]. The shortest and the longest ESTs were ~200 and ~2200 bp, respectively. PCR products were sequenced using the GeneRacer 5' primer. Plasmid and adaptor sequences were manually removed using the BioEdit program. The CAP3 program [x01] assembled all ESTs to 217 unique sequences (so called "unigenes"): 137 singletons and 80 contigs. Each contig was derived from multiple clones, with 2 to 37 clones per unigene [Figure 1]. Of the 217 unigenes, 68% (n=147) appear to represent full-length transcripts, because they contained both the GeneRacer RNA oligo sequence (which is attached to the 5' end of full-length mRNAs) and poly-A tail.

Gene diversity of *P. insidiosum*

The *P. insidiosum* unigene sequences were annotated using ESTexplorer [x02, x03]. Although 73 unigenes (34%) did not match any protein in the database, 144 unigenes (66%) had significant hits (E-value < -4): 47 unigenes (22%) encoding ribosomal proteins and 97 unigenes (44%) encoding non-ribosomal proteins, including 9 hypothetical proteins [Table 1]. In addition, 13 of 73 unigenes with no significant BLASTX hits and 2 of 9 unigenes matched to a hypothetical proteins were assigned a protein domain by InterProScan [x32] [Table 2]. Thirty highly-representative unigenes (at least 4 clones per unigene) are shown in Table 3. Several sets of similar genes (gene families) were identified: 47 unigenes encode ribosomal proteins, 7 encode elicitin-like proteins (ELLs), 3 encode peptidylprolyl isomerases, and 2 each encode eukaryotic translation initiation factors, histones, RNA polymerases, small nuclear riboproteins, thioredoxin-like proteins, ubiquitins, and V-type ATPase proteolipid subunits.

The BLAST2GO program [x33] assigned 90 GO terms to 60 annotated unigenes. which can be divided into 3 categories: (i) Biological processes (37.8%), including proteins involved in cell organization and biogenesis, transport, nucleic acid binding, protein metabolism, developmental processes, cell cycle and proliferation, RNA metabolism, signal transduction and RNA transcription; (ii) Cellular components (28.9%), including proteins associated with specific sub-cellular fractions, including internal or plasma membrane, ribosome, mitochondrion, nucleus, cytosol, cytoskeleton, and vacuole; and (iii) Molecular function (33.3%), including transcription regulation, ribosome binding, kinase activity and transport. The KOBAS program [x34] indicated that 21 non-ribosomal protein-encoding genes are in 11 metabolic pathways: polyunsaturated fatty acid biosynthesis (PinsEST#104 and 146), methionine metabolism (PinsEST#037, 141 and 215), styrene degradation (PinsEST#103), tyrosine metabolism (PinsEST#074, 078 and 103), selenoamino acid metabolism (PinsEST#037, 141 and 215), chaperones and folding catalysts (PinsEST#067, 156, 158 and 200), protein folding and associated processing (PinsEST#016, 197 and 209), pyrimidine metabolism (PinsEST#021, 034 and 114), nitrogen metabolism (PinsEST#027 and 158), small cell lung cancer (PinsEXT#096), and C5-branched dibasic acid metabolism (PinsEST#069).

The SignalP 3.0 program [x04], with the criteria set by Win et al. [x05], predicts that 20 unigenes encode extracellular (secreted) proteins. Nine of them (PinsEST#007, 014, 026, 050, 072, 083, 084, 153 and 217) had no BLASTX hits, while the rest (n=11) include seven putative *P. insidiosum* ELLs (PinsEST#003, 025, 042, 045, 046, 066 and 176), two hypothetical proteins (PinsEST#149 and 151), and one each of an Os09g0542200-similar protein (PinsEST#052) and a cellulose binding elicitor lectin (CBEL; PinsEST#112).

Putative pathogenicity genes of *P. insidiosum*

Based on sequence homology to virulence genes of other pathogens, a handful of *P. insidiosum* genes encode putative virulence factors [Table 4]. These include ELLs, CBEL, and RXLR effector, which are pathogenicity proteins of plant-pathogenic oomycetes [x17, x47, x45]. Other *P. insidiosum* genes are predicted to encode enzymes with antioxidant functions, which can be important for pathogen survival in response to radical oxygen species produced by host organisms. These include superoxide dismutase [x10], thioredoxin-like protein [x15], and glutaredoxin [x11]. Predicted genes for calmodulin [x08] and heat shock transcription factor [x13] may facilitate growth and thermal adaptation at the animal host body temperature (37°C). There is a predicted *P. insidiosum* gene for β-carbonic anhydrase [x09]. This enzyme could promote a superficial infection on host surface (i.e., eye, skin) where CO_2 content is low. Finally, there are predicted genes with homology to macrophage migration inhibitory factor [x14] and ferrochelatase, an enzyme required for the final step in heme biosynthesis and is important for microbial virulence [x12].

Putative effectors of *P. insidiosum*

Effectors are a group of secreted molecules that manipulate host cell structure and function, in order to facilitate infection processes of many pathogens, including oomycetes [x68]. We identified *P. insidiosum* genes that encode effectors, including ELI or ELL, CBEL, and RXLR homoloques. PinsEST#112 significantly matched the *P. parasitica* CBEL surface protein (E-value is 1.02E-09), and a "Conserved Domain Search" (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) revealed domains shared with the *P. parasitica* CBEL, including a signal peptide, and repeats of the PAN/Apple domain (four copies in PinsEST#112 vs. two in *P. parasitica*) [Figure 2]. In contrast, PinsEST#112 lacks the cellulose-binding domains of *P. parasitica* CBEL [Figure 2]. Since cell wall associated proteins are often O- and N-glycosylated, we used the NetOGlyc and NetNGlyc software [http://www.cbs.dtu.dk/services/NetNGlyc/] to predict O- and N-glycosylation sites and found two asparagine residuals (position 76 and 91) of PinsEST#112 protein with the predicted potential as N-glycosylation sites.

ELIs share the highly-conserved 98-amino acid elicitin domain with six cysteine residues, while ELLs contain either shorter or longer domains, with more diverse sequences [x17]. Seven ELLs were identified by BLASTX (n=6) and InterProScan (n=1) search, and were named INSL1 (PinsEST#003), INSL2 (PinsEST#025), INSL3 (PinsEST#042), INSL4 (PinsEST#045), INSL5 (PinsEST#046), INSL6 (PinsEST#066), and INSL7 (PinsEST#176). The mean protein length of all *P. insidiosum* ELLs, was 133 amino acids (range, 112 – 181), signal peptides were 16-20 amino acids long, the elicitin domain (Pfam PF00964) varied from 66 – 96 amino acids, and C-terminal domains were 5 – 51 amino acids long [Figure 2]. Only INSL2 and INSL7 had predicted O-glycosylation sites (20 and 13 sites, respectively). The big-PI Fungal Predictor [x36] identified no glycosylphosphatidylinositol (GPI) anchor in any *P. insidiosum* ELLs. The elicitin domain sequences of all *P. insidiosum* ELLs were aligned with that of some other oomycetes, such as, *Phytophthora cryptogea* (CRY; assession number, P15570.2), *Pythium vexans* (VEX1; assession number, AAB34416.1), *Phytophthora brassicae* (BRA2; assession number, AAO92424.1), *P. sojae* (SOJB; assession number, AAO24640.1), *P. ramorum* (RAM3B; assession number, ABB55984.1), and *P. infestans* (INF5; assession number, AAL16012.1) [Figure 4]. The alignment shows that all *P. insidiosum* ELLs have the six conserved cysteine residues, a main characteristic of elicitins (ELIs) for the predicted formation of 3 disulfide bonds [x49, x17] [Figure 4].

Structurally, an RXLR effector has an N-terminus portion that comprises a signal peptide (for secretion of effector), an RXLR-dEER domain (for translocation of effector), and a C-terminus functional domain, necessary for pathogenicity [x18, x19, x68]. The RXLR motif usually resides between the position 31 and 58, while that of the dEER motif is between positions 51 and 86 [x20, x21]. The dEER motif is less conserved than the RXLR motif, and characterized by a stretch of mainly acidic amino acids (D/E) [x18, x19]. To find a putative RXLR effector of *P. insidiosum*, the string "RXLR" was initially searched in all 217 predicted protein sequences. Proteins with this RXLR motif were then searched for the signal peptide and dEER motif. A total of 12 proteins contained the RXLR motif within the first 60 amino acids of the N-terminus. Two of these (PinsEST#107 and 113) had both RXLR and dEER motifs, but no predicted signal peptide. Only one protein (PinsEST#176) had a signal peptide and the RXLR-dEER domain. The gene products of PinsEST#107 and 113 had no BLAST hit, whereas that of PinsEST#176 was described above as an ELL (INSL7). The RXLR-dEER domains from INSL7 and four prototypic oomycete RXLR effectors (Avr1b from *P. sojae* [x79], Avr3a from *P. infestans* [x80], and ATR1 and ATR13 from *Hyaloperonospora parasitica* [x77, x78]) are aligned in Figure 3. All sequences have the conserved RXLR motif, but diverse dEER motif sequences. Similar to ATR13, the INSL7 has only one acidic amino acid (E) in the dEER motif.

Phylogenetic analysis of *P. insidiosum* elicitins

Jiang et. al. [x17] reported that 156 elicitin domain sequences from *Phytophthora* and *Pythium* species can be phylogenetically divided into 17 distinct clades: four ELIs and 13 ELLs. The elicitin domain sequences from all 7 ELLs (INSL1-7) of *P. insidiosum* and 38 ELIs/ELLs of other oomycetes [x17] were used to construct an unrooted phylogenetic tree based on Neighbor-Joining analysis with 1,000 bootstrap replicates. Analyses using the programs PHYLIP, TreeView, BioNJ and TreeDyn via Phylogeny.fr provided similar trees (Figure 5). Bootstrap scores $\geq$ 70% were present at each corresponding node. All of the elicitin domain sequences, except six from *P. insidiosum* (INSL1-6), fell into the 17 clades, as reported by Jiang et al. [x17]. Of all seven *P. insidiosum* ELLs, INSL7 was in clad ELL-06, and the rest, INSL1-6, were in novel clades: INSL1, INSL3 and INSL6 located in clade ELL-A, ELL-C and ELL-D, respectively, while INSL2, INSL4, and INSL5 formed clade ELL-B.

Predicted structure of *P. insidiosum* elicitins

Sequence alignment of 13 elicitin domain sequences, including that of the structure- and function-defined elicitin CRY [x37], showed up to 16 possible ergosterol interactive sites in the elicitin domains of *P. insidiosum* ELLs [Figure 4]. Using homology modeling based on the structure-defined elicitin 1bxm [x37] and 2pos [x52], all *P. insidiosum* elicitins, except INSL7, have predicted three-dimentional structures with a small pocket-like cavity. Protein-ligand docking analysis using SwissDock (see the methods for

details) predicts that three *P. insidiosum* elicitins (INSL1, INSL2 and INSL5) could encapsulate either cholesterol or ergosterol in the cavity. Predicted structures of the complex between INSL2 and cholesterol are depicted in Figure 6.

EST comparative analysis

BLASTX and TBLASTX were used to search protein databases from the genomes of six oomycete species: *P. ultimum*, *P. sojae*, *P. ramorum*, *P. infestans*, *H. arabidopsidis* and *S. parasitica*. These species were chosen because of the availability of their genome sequences. While most of our ESTs showed homology to other sequences in these oomycete databases, we found one putative gene, with unknown function, that is unique to *P. insidiosum* (PinsEST#086). Orthologs of PinsEST#119 are only found in *P. infestans* (AAY43427.1) and *S. parasitica* (SPRG_10907) genomes. Sequence alignment and domain analyses showed that PinsEST#119 and its *S. parasitica* orthologs contain a domain of the *Rickettsia* 17-kDa surface antigen, while that of *P. infestans* does not (data not shown).

DISCUSSION

The present study reports the first molecular genetics insights into the human pathogenic oomycete, *P. insidiosum*, through EST analysis. Approximately, 500 *P. insidiosum* ESTs were generated from young mycelium actively grown at body temperature (37 °C). As implied by the size of ESTs, most genes of *P. insidiosum* obtained were small- to medium-size genes (83% were 400 – 900 bp in length). The ESTs can be assembled into 217 unigenes. BLASTX searches demonstrated that 66% (n=144) of the unigenes matched with annotated proteins. Not surprisingly, one third of these (n=49) encode ribosomal proteins. The remaining annotated matches (two thirds; n=95) include proteins with roles in a broad spectrum of biological processes, molecular functions or cellular composition [Table 1]. Not unexpectedly, 34% (n=73) of all unigenes did not match any proteins in the NCBI database, indicating that these genes are unique to *P. insidiosum* or their orthologs have not been reported yet from other organisms. These unmatched predicted proteins were further analyzed with InterProScan, and possible functional domains were assigned to 15 proteins [Table 2]. In addition, 10 unigenes, with no BLASTX hit, were predicted to encode secreted proteins by SignalP [x72].

Based on the EST sequences, sixteen putative proteins of *P. insidiosum* have homology to virulence factors of some fungal, parasitic, and bacterial pathogens of humans, animals and plants [Table 4]. Three *P. insidiosum* genes involved in oxidative stress response were identified: copper-zinc superoxide dismutase [x10], thioredoxin [x15], and glutaredoxin [x11]. In *Cryptococcus neoformans*, the gene *SOD1* (encoding copper-zinc superoxide dismutase) is essential to protect the pathogen from the toxic effects of reactive oxygen intermediates and subsequent production of toxic hydroxyl radicals. Glutaredoxins (or glutathione reductases) are ubiquitous cytosolic enzymes that catalyze reduction of glutathione disulphide. In *Candida albicans*, the glutaredoxin gene *GRX2* is upregulated in response to either polymorphonuclear cells or H_2O_2 challenge. Also in *C. albicans*, the thioredoxin gene, *Trx1*, encodes a protein with antioxidative activity and also plays a central role in coordinating the response of the pathogen to oxidative stress [x15]. The capacity to respond to reactive oxygen species (ROS) is essential for survival of the systemic fungal pathogens in the host. Unsurprisingly, disruption of a gene encoding each of these antioxidant enzymes (SOD1, GRX2 or Trx1) leads to attenuation of virulence of these pathogens [x10, x11, x15].

It is important for a successful pathogen to retain its physiological balance against not only ROS, but some other host stress conditions, such as, high body temperature (37°C), limited essential nutrients, and host immune responses. Our EST dataset contains calmodulin [x08] and heat shock transcription factor [x13] both of which play a role in growth and thermal adaptation of pathogens inside the host. For example, upon sensing and binding to Ca^{2+}, the *C. neoformans* calmodulin CAM1 undergoes conformational change and then activates the heterodimeric calcineurin, which is required for growth at high temperature. Disruption of the *C. neoformans CAM1* shows abnormal morphogenesis and impaired growth at body temperature [x08]. The heat shock transcription factor gene *Hsf1* of *C. albicans* is an essential gene and plays a central role in thermal adaptation by activation of chaperones in response to increased temperature [x13]. When the CE2 domain of Hsf1 was mutated, the pathogen remained viable, but was sensitive to body temperature and lost virulence. Another EST-encoded protein with possible involvement in virulence for *P. insidiosum* is β-carbonic anhydrase. When the carbonic anhydrase-encoding gene *NCE103* of *C. albicans* was disrupted, the pathogen failed to grow and invade tissue at low CO_2, (0.033%, which is equivalent to atmospheric CO_2 content) [x09]. In contrast, when the mutant strain is supplied with high CO_2 content (5%, which is equivalent to CO_2 content inside hosts), it can grow and invade tissue. These findings suggest a possible role for carbonic anhydrase in low CO_2 conditions, such as in superficial infections of

the skin and eye.

In the hosts, a diverse set of innate and adaptive immune responses is employed to guard against various pathogens. In the malaria parasite *Plasmodium falciparum*, a homolog of human macrophage migration inhibitory factor, called *P. falciparum* macrophage migration inhibitory factor (PfMIF), plays a role in modification of host immunity [x14]. For example, the recombinant PfMIF inhibits the migration of monocytes. Expressions of some Toll-like receptors (i.e., TLR2 and TLR4) on monocytes are significantly reduced in response to PfMIF. It will be of interest to determine what role the identified PfMIF homolog of *P. insidiosum* plays during infections.

P. insodiosum causes both deep tissue (i.e., artery and some other internal organs) and superficial (i.e., skin and eye) infections, and therefore, must adapt itself to high temperature, low CO_2 content, and host immune responses. *P. insidiosum* genes encoding putative calmodulin, heat shock transcription factor, carbonic anhydrase and macrophage migration inhibitory factor could be very important for ensuring a well-being of the pathogen inside its hosts, as well as, for participating in the pathogenesis process.

P. insidiosum was found to express a gene encoding ferrochelatase. In both prokaryotes and eukaryotes, this enzyme is required for the final step of the heme biosynthesis by catalyzing the introduction of an iron molecule into porphyrin [x12]. Since unbound iron is toxic to cells, a microbial heme could bind and store iron as a source for further metabolic uses [x44]. Heme is a tightly-bound cofactor of enzymes responsible for oxygen transport, production of energy, and signal transduction. In the pathogenic bacterium *Brucella abortus*, which causes the infectious disease "brucellosis" in humans and animals, ferrochelatase is necessary for survival inside the host [x12]. Deletion of the ferrochelatase-encoding gene *hemH* transforms *B. abortus* to hemin auxotrophy and attenuates its virulence. Of particular interest for *P inidiosum* infections in regard to iron metabolism is that a major predisposing factor for pythiosis is thalassemia. Since the majority of pythiosis patients are in a state of iron overload as a result of the thalassemia and associated blood transfusions used for treatment [x62, x73], it will be interesting to explore the roles of the putative ferrochelatase of *P. insidiosum* in iron metabolism and virulence.

Many pathogens produce secretory molecules, so called effectors, which can manipulate host cell structure and function in order to facilitate the infection processes [x68]. Based on target sites, effectors can be simply divided into two groups: extracellular (or apoplastic in the case of plant pathogens) effectors which are secreted to interact with extracellular targets, and intracellular (or cytoplasmic) effectors which are secreted and translocated inside host cells [x68]. Several *P. insidiosum* genes had sequences similar to extracellular (elicitin [x17] and CBEL [x45]) and intracellular (RXLR [x19]) effectors of the plant-pathogenic oomycetes. The adhesion molecule CBEL was first described in the plant-pathogenic oomycete *P. parasitica* [x45]. It has two tandem repeats of cellulose-binding domain and PAN/apple domain [Figure 2]. It is a potent inducer of necrosis and defense-gene expression during invasion of its host plant [x45]. Interestingly, the *P. parasitica* CBEL can agglutinate human red blood cells, indicating it has lectin-like activity. Although the PinsPAN1 (PinsEST#112) of *P. insidiosum* has a high degree of sequence similarity to the *P. parasitica* CBEL, it does not contain any cellulose-binding domain, but has four tandem repeats of the PAN/apple domain [Figure 2]. The apicomplexan parasite *Toxoplasma gondii* produces micronemal proteins (i.e., MIC1, MIC2, MIC3 and MIC4), which are involved in host cell attachment and penetration [x46]. It has been shown that MIC4 possesses 6 copies of the PAN/apple domain, one of which is required for efficient host cell binding [x46] (Figure 2). These results suggest possible roles for the PinsPAN1 of *P. insidiosum* in host cell attachment and intravascular hemagglutination, processes responsible for occlusion of affected arteries.

So far, ELIs and ELLs have been found only in *Phytophthora* and some *Pythium* species, and form a large protein family with the highly-conserved elicitin domain [x17, x48]. In host plants, ELIs trigger defense responses and programmed cell death in the so-called hypersensitive response. For some interactions, this response is thought to promote infection by some pathogens that colonize the dying plant tissue [x17, x48]. Expression of ELLs can be stage specific, as seen for the prototypic elicitin, INF1, of *P. infestans* that is highly expressed in mycelium, but is not found at other growth stages, such as in the zoospore or cyst [x47]. The sequences of elicitin domains derived from 156 ELIs and ELLs of *Phytophthora* and *Pythium* species are phylogenetically categorized into 17 clades (ELI1-4 and ELL1-13) [x17]. Sequences of all elicitin domains from *P. insidiosum* and 38 elicitins from other oomycetes were used to construct a phylogenetic tree [Figure 5]. All, except one (INSL7 in clad ELL6), of the *P. insidiosum* elicitins locate in several novel clades (namely, ELL-A, -B, -C, and -D), indicating divergence of *P. insidiosum* elicitins from those of other oomycetes. However, in the phylogenetic distribution in Figure 5, as well as in previously published studies, the various ELIs and ELLs of *Phytophthora* and *Pythium* are

intermixed across the tree, suggesting that the diversification began before divergence of these species from a common ancestor [x17, x26].

Based on the clone frequency in the EST library, the *P. insidiosum* elicitins, especially INSL1 (PinsEST#003), INSL2 (PinsEST#025) and INLS4 (PinsEST#045), are highly expressed in young mold cultures [Table 3], suggesting that their function is important for the pathogen. Biochemical studies and the X-ray crystal structures of elicitin-ergosterol complexes indicate that ELIs can function as sterol carrier proteins [x37]. This sterol-carrying function of ELIs is thought to be an alternative mechanism for acquiring exogenous sterols for growth of *Phytophthora* and *Pythium* species, which lack enzymes for endogenous ergosterol synthesis [x30, x50, x51]. This is consistent with the fact that use of antifungal drugs targeting the ergosterol synthesis pathway fails to control *P. insidiosum* infections [x62]. Model predictions based on protein-ligand docking analysis showed that some *P. insidiosum* elicitins should bind, not only ergosterol, but also cholesterol (an abundant human/animal sterol) in the pocket-like cavity [Figure 6]. Whether or not *P. insidiosum* can synthesize its own sterol, the discovery of the sterol-carrier, elicitins, could explain why the pathogen can grow against the effects of conventional antifungal drugs. The prediction of many O-glycosylation sites of INSL2 and INSL7 suggests that these *P. insidiosum* ELLs are cell wall-associated proteins, whereas the rest (INSL1 and INSL3-6), which contained neither O-glycosylation nor GPI anchor site, are predicted to function extracellularly. The ELIs from plant-pathogenic oomycetes are pathogen-associated molecular patterns (or PAMPs) [x85] and they stimulate plant host defenses [x17, x48]. Further experimentation will be required to reveal the biological and pathogenesis roles of the ELLs in *P. insidiosum* interaction with human hosts.

In recent years, advances have been made on the elucidation of the role of RXLR effectors of the plant-pathogenic oomycetes [x19, x68, x18, x74, x25]. Well-studied RXLR effectors include Avr3a of *P. infestans* [x80], Avr1b of *P. sojae* [x79], and ATR1 and ATR13 of *H. parasitca* [x77, x78]. These effectors are secreted into extracellular space and translocated into the host cell cytosol (via the RXLR-dEER domain) where they alter host responses (via the functional domain in the C-terminus) [x19, x18, x25, x76] (Figure 2). Unlike the type III secretion system of pathogenic bacteria, which requires a number of pore-forming molecules to facilitate entry of its effector into host cells [x18], the RXLR-dEER domain is solely responsible for translocation of the effector molecule, without any help from other pathogen molecules [x75]. This function of the RXLR-dEER domain mimics the PEXEL domain (which also contains an RXLX motif) of many *P. falciparum* effectors, such as, the knob-associated histidine-rich protein, KAHRP (Figure 2) [x81, x82, x83]. The parasite employs the PEXEL to transfer its secreted effectors across the parasitophorous vacuole membrane to the erythrocyte cytosol as part of the infection cycle. The oomycete RXLR domain and the *P. falciparum* PEXEL are functionally interchangeable [x19, x18, x25], suggesting a conserved mechanism for translocation. While the genomes of most oomyctes studied to date encode many RXLR effectors, that of *P. ultimum*, the only complete genome sequence of a *Pythium* species available, does not encode any RXLR-containing proteins [x23]. In addition, the large-scale EST database of *Aphanomyces euteiches* [x30] does not contain RXLR effectors. These results suggest that RXLR effectors are not present in all oomycete pathogens, and that the pathogenic strategy using RXLR effectors is lost in some oomycetes. The *P. insodiosum* EST–encoded protein, INSL7, contains a putative RXLR-dEER domain, shortly following a predicted signal peptide [Figure 2], and the RXLR motif of INSL7 is flanked by a few acidic amino acid residues (similar to the prototypic RXLR effector ATR13) [Figure 3]. In addition, an elicitin domain was found in the C-terminus of INSL7 [Figure 2]. Future works should address whether the INSL7 is a functional RXLR effector that is secreted by *P. insodiosum*, enters host cells, and thereby promotes the infection process.

Among the pathogenic oomycetes, *P. insidiosum* is the only one that infects humans. It is still a mystery as to what molecules and strategies *P. insidiosum* has developed to be a successful pathogen of humans. Identification of *P. insidiosum* genes that are absent in other saprophytic or pathogenic oomycetes may give some genetic clues to address this important question. Here, we performed an initial comparison between *P. insidiosum* ESTs and the ESTs and/or genomes of 6 other oomycetes. Although most of the *P. insidosum* genes have homology to sequences of other oomycetes, one gene with unknown function, PinsEST#086, was found to be unique to *P. insidiosum*. We also found that orthologs of PinsEST#119 are only present in *P. infestans* and *S. parasitica*. So, while several intriguing genes have been identified as possible virulence factors, the current *P. insidiosum* EST dataset is too small for comprehensive comparative analyses among the saprophytic and pathogenic oomycetes. It will be necessary to generate a much larger set of ESTs, as well as the genome sequence, of *P. insidiosum* to provide a thorough elucidation of its pathogenic processes and their evolution.

The main clinical concern for pythiosis, with its high morbidity and mortality rates, is the lack of an effective medical treatment, such as, antimicrobial drug and vaccine. A better understanding of

pathogenic mechanisms should lead to better methods of infection control. We report here the first EST dataset of this orphan pathogen. The information gained from these studies, while limited, already provides many potentially useful avenues for further research, toward the long-term goal of discovering new methods for treatment of pythiosis.

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REFERENCES

x01. Huang, X. and Madan, A. (1999) CAP3: A DNA Sequence Assembly Program. Genome Research, 9: 868-877.

x02. Nagaraj SH, Deshpande N, Gasser RB, Ranganathan S. ESTExplorer: an expressed sequence tag (EST) assembly and annotation platform. Nucleic Acids Res. 2007 Jul;35(Web Server issue):W143-7. Epub 2007 Jun 1.

x03. Altschul,S.F., Madden,T.L., Schaffer,A.A., Zhang,J., Zhang,Z., Miller,W. and Lipman,D.J. (1997) Gapped BLAST and PSIBLAST: a new generation of protein database search programs. Nucleic Acids Res., 25, 3389–3402.

x04. Bendtsen JD, Nielsen H, von Heijne G, Brunak S. Improved prediction of signal peptides: SignalP 3.0. J Mol Biol. 2004 Jul 16;340(4):783-95.

x05. Win J, Kanneganti TD, Torto-Alalibo T, Kamoun S. Computational and comparative analyses of 150 full-length cDNA sequences from the oomycete plant pathogen Phytophthora infestans. Fungal Genet Biol. 2006 Jan;43(1):20-33.

x06. Zhi-Liang Hu, Jie Bao and James M. Reecy (2008) "CateGOrizer: A Web-Based Program to Batch Analyze Gene Ontology Classification Categories". Online Journal of Bioinformatics. 9 (2):108-112.

x07. Wang P, Cardenas ME, Cox GM, Perfect JR, Heitman J. Two cyclophilin A homologs with shared and distinct functions important for growth and virulence of *Cryptococcus neoformans*. EMBO Rep. 2001 Jun;2(6):511-8.

x08. Peter R. Kraus, Connie B. Nichols, and Joseph Heitman. Calcium- and Calcineurin-Independent Roles for Calmodulin in *Cryptococcus neoformans* Morphogenesis and High-Temperature Growth. Eukaryot Cell. 2005 June; 4(6): 1079–1087.

x09. Klengel T, Liang WJ, Chaloupka J, Ruoff C, Schröppel K, Naglik JR, Eckert SE, Mogensen EG, Haynes K, Tuite MF, Levin LR, Buck J, Mühlschlegel FA. Fungal adenylyl cyclase integrates CO2 sensing with cAMP signaling and virulence. Curr Biol. 2005 Nov 22;15(22):2021-6.

x10. Narasipura SD, Ault JG, Behr MJ, Chaturvedi V, Chaturvedi S. Characterization of Cu,Zn superoxide dismutase (SOD1) gene knock-out mutant of *Cryptococcus neoformans* var. *gattii*: role in biology and virulence. Mol Microbiol. 2003 Mar;47(6):1681-94.

x11. Chaves GM, Bates S, Maccallum DM, Odds FC. *Candida albicans* GRX2, encoding a putative glutaredoxin, is required for virulence in a murine model. Genet Mol Res. 2007 Oct 5;6(4):1051-63.

x12. Almirón M, Martínez M, Sanjuan N, Ugalde RA. Ferrochelatase is present in *Brucella abortus* and is critical for its intracellular survival and virulence. Infect Immun. 2001 Oct;69(10):6225-30.

x13. Nicholls S, Maccallum DM, Kaffarnik FA, Selway L, Peck SC, Brown AJ. Activation of the heat shock transcription factor Hsf1 is essential for the full virulence of the fungal pathogen *Candida albicans*. Fungal Genet Biol. 2010 Sep 9. [Epub ahead of print]

x14. Cordery, D. V., U. Kishore, S. Kyes, M. J. Shafi, K. R. Watkins, T. N. Williams, K. Marsh, and B. C. Urban. 2007. Characterization of a *Plasmodium falciparum* macrophage migration inhibitory factor homologue. J. Infect. Dis. 195:905-912

x15. da Silva Dantas A, Patterson MJ, Smith DA, Maccallum DM, Erwig LP, Morgan BA, Quinn J. Thioredoxin regulates multiple hydrogen peroxide-induced signaling pathways in *Candida albicans*. Mol Cell Biol. 2010 Oct;30(19):4550-63.

x16. Gaulin E, Dramé N, Lafitte C, Torto-Alalibo T, Martinez Y, Ameline-Torregrosa C, Khatib M, Mazarguil H, Villalba-Mateos F, Kamoun S, Mazars C, Dumas B, Bottin A, Esquerré-Tugayé MT, Rickauer M. Cellulose binding domains of a Phytophthora cell wall protein are novel pathogen-associated molecular patterns. Plant Cell. 2006 Jul;18(7):1766-77.

x17. Jiang RH, Tyler BM, Whisson SC, Hardham AR, Govers F. Ancient origin of elicitin gene clusters in Phytophthora genomes. Mol Biol Evol. 2006 Feb;23(2):338-51.

x18. Govers F, Bouwmeester K. Effector trafficking: RXLR-dEER as extra gear for delivery into plant cells. Plant Cell. 2008 Jul;20(7):1728-30.

x19. Morgan W, Kamoun S. RXLR effectors of plant pathogenic oomycetes. Curr Opin Microbiol. 2007 Aug;10(4):332-8.
x20. Win J, Kamoun S. Adaptive evolution has targeted the C-terminal domain of the RXLR effectors of plant pathogenic oomycetes. Plant Signal Behav. 2008 Apr;3(4):251-3.
x21. Win J, Morgan W, Bos J, Krasileva KV, Cano LM, Chaparro-Garcia A, Ammar R, Staskawicz BJ, Kamoun S. Adaptive evolution has targeted the C-terminal domain of the RXLR effectors of plant pathogenic oomycetes. Plant Cell. 2007 Aug;19(8):2349-69.
x22. Tyler BM, Tripathy S, Zhang X, Dehal P, Jiang RH, Aerts A, Arredondo FD, Baxter L, Bensasson D, Beynon JL, Chapman J, Damasceno CM, Dorrance AE, Dou D, Dickerman AW, Dubchak IL, Garbelotto M, Gijzen M, Gordon SG, Govers F, Grunwald NJ, Huang W, Ivors KL, Jones RW, Kamoun S, Krampis K, Lamour KH, Lee MK, McDonald WH, Medina M, Meijer HJ, Nordberg EK, Maclean DJ, Ospina-Giraldo MD, Morris PF, Phuntumart V, Putnam NH, Rash S, Rose JK, Sakihama Y, Salamov AA, Savidor A, Scheuring CF, Smith BM, Sobral BW, Terry A, Torto-Alalibo TA, Win J, Xu Z, Zhang H, Grigoriev IV, Rokhsar DS, Boore JL. Phytophthora genome sequences uncover evolutionary origins and mechanisms of pathogenesis. Science. 2006 Sep 1;313(5791):1261-6.
x23. Lévesque CA, Brouwer H, Cano L, Hamilton JP, Holt C, Huitema E, Raffaele S, Robideau GP, Thines M, Win J, Zerillo MM, Beakes GW, Boore JL, Busam D, Dumas B, Ferriera S, Fuerstenberg SI, Gachon CM, Gaulin E, Govers F, Grenville-Briggs L, Horner N, Hostetler J, Jiang RH, Johnson J, Krajaejun T, Lin H, Meijer HJ, Moore B, Morris P, Phuntmart V, Puiu D, Shetty J, Stajich JE, Tripathy S, Wawra S, van West P, Whitty BR, Coutinho PM, Henrissat B, Martin F, Thomas PD, Tyler BM, De Vries RP, Kamoun S, Yandell M, Tisserat N, Buell CR. Genome sequence of the necrotrophic plant pathogen Pythium ultimum reveals original pathogenicity mechanisms and effector repertoire. Genome Biol. 2010;11(7):R73.
x24. Haas BJ, Kamoun S, Zody MC, Jiang RH, Handsaker RE, Cano LM, Grabherr M, Kodira CD, Raffaele S, Torto-Alalibo T, Bozkurt TO, Ah-Fong AM, Alvarado L, Anderson VL, Armstrong MR, Avrova A, Baxter L, Beynon J, Boevink PC, Bollmann SR, Bos JI, Bulone V, Cai G, Cakir C, Carrington JC, Chawner M, Conti L, Costanzo S, Ewan R, Fahlgren N, Fischbach MA, Fugelstad J, Gilroy EM, Gnerre S, Green PJ, Grenville-Briggs LJ, Griffith J, Grünwald NJ, Horn K, Horner NR, Hu CH, Huitema E, Jeong DH, Jones AM, Jones JD, Jones RW, Karlsson EK, Kunjeti SG, Lamour K, Liu Z, Ma L, Maclean D, Chibucos MC, McDonald H, McWalters J, Meijer HJ, Morgan W, Morris PF, Munro CA, O'Neill K, Ospina-Giraldo M, Pinzón A, Pritchard L, Ramsahoye B, Ren Q, Restrepo S, Roy S, Sadanandom A, Savidor A, Schornack S, Schwartz DC, Schumann UD, Schwessinger B, Seyer L, Sharpe T, Silvar C, Song J, Studholme DJ, Sykes S, Thines M, van de Vondervoort PJ, Phuntumart V, Wawra S, Weide R, Win J, Young C, Zhou S, Fry W, Meyers BC, van West P, Ristaino J, Govers F, Birch PR, Whisson SC, Judelson HS, Nusbaum C. Genome sequence and analysis of the Irish potato famine pathogen Phytophthora infestans. Nature. 2009 Sep 17;461(7262):393-8.
x25. Tyler BM. Entering and breaking: virulence effector proteins of oomycete plant pathogens. Cell Microbiol. 2009 Jan;11(1):13-20.
x26. Kamoun, S., Hraber, P., Sobral, B., Nuss, D., and Govers, F. (1999) Initial assessment of gene diversity of the oomycete plant pathogen Phytophthora infestans. Fungal Genet Biol 28: 94–106.
x27. Qutob, D., Hraber, P., Sobral, B., and Gijzen, M. (2000) Comparative analysis of expressed sequences in *Phytophthora sojae*. *Plant Physiol* **123:** 243–253.
x28. Randall, T.A., Dwyer, R.A., Huitema, E., Beyer, K., Cvitanich, C., Kelkar, H., *et al.* (2005) Large-scale gene discovery in the oomycete *Phytophthora infestans* reveals likely components of phytopathogenicity shared with true fungi. *Mol Plant Microbe Interact* **18:** 229–243.
x29. Torto-Alalibo, T., Tian, M., Gajendran, K., Waugh, M.E., van West, P., and Kamoun, S. (2005) Expressed sequence tags from the oomycete fish pathogen *Saprolegnia parasitica* reveal putative virulence factors. *BMC Microbiol* **5:** 46.
x30. Gaulin, E., Madoui, M.A., Bottin, A., Jacquet, C., Mathe, C., Couloux, A., *et al.* (2008) Transcriptome of *Aphanomyces euteiches*: new oomycete putative pathogenicity factors and metabolic pathways. *PLoS ONE* **3:** e1723.
x31. Le Berre, J.Y., Engler, G., and Panabieres, F. (2008) Exploration of the late stages of the tomato–*Phytophthora parasitica* interactions through histological analysis and generation of expressed sequence tags. *New Phytol* **177:** 480–492.
x32. Quevillon,E., Silventoinen,V., Pillai,S., Harte,N., Mulder,N., Apweiler,R. and Lopez,R. (2005) InterProScan: protein domains identifier. Nucleic Acids Res., 33, W116–W120.
x33. Conesa,A., Gotz,S., Garcia-Gomez,J.M., Terol,J., Talon,M. and Robles,M. (2005) Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. Bioinformatics, 21, 3674–3676.
x34. Wu,J., Mao,X., Cai,T., Luo,J. and Wei,L. (2006) KOBAS server: a web-based platform for automated annotation and pathway identification. Nucleic Acids Res., 34, W720–W724.
x35. Julenius K, Mølgaard A, Gupta R, Brunak S. Prediction, conservation analysis,and structural characterization of mammalian mucin-type O-glycosylation sites.Glycobiology. 2005 Feb;15(2):153-64.

x36. Eisenhaber B, Schneider G, Wildpaner M, Eisenhaber F. A sensitive predictor for potential GPI lipid modification sites in fungal protein sequences and its application to genome-wide studies for Aspergillus nidulans, Candida albicans, Neurospora crassa, Saccharomyces cerevisiae and Schizosaccharomyces pombe. J Mol Biol. 2004 Mar 19;337(2):243-53.
x37. Boissy, G., M. O└ Donohue, O. Gaudemer, V. Perez, J. C. Pernollet, and S. Brunie. 1999. The 2.1 Å structure of an elicitin-ergosterol complex: a recent addition to the sterol carrier protein family. Protein Sci. 8:1191–1199.
x38. Felsenstein, J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution. 39:783–91.
x39. Thompson J.D., Higgins D.G., Gibson T.J. *CLUSTAL W: improving the sensitivity of progressivemultiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice.* Nucleic Acids Res. 1994, Nov 11;22(22):4673-80.
x40. Page, R.D.M. 1996. TREEVIEW: an application to display phylogenetic trees on personal computers. Compl. Appl. Biosci. 12:357–358.
x41. Dereeper A.*, Guignon V.*, Blanc G., Audic S., Buffet S., Chevenet F., Dufayard J.F., Guindon S., Lefort V., Lescot M., Claverie J.M., Gascuel O. *Phylogeny.fr: robust phylogenetic analysis for the non-specialist.* Nucleic Acids Res. 2008 Jul 1;36(Web Server issue):W465-9.
x42a. Felsenstein J. *PHYLIP - Phylogeny Inference Package (Version 3.2).* 1989, Cladistics 5: 164-166
x42b. Gascuel O. *BIONJ: an improved version of the NJ algorithm based on a simple model of sequence data.* Mol Biol. Evol. 1997, Jul;14(7):685-95.
x43. Chevenet F., Brun C., Banuls AL., Jacq B., Chisten R. *TreeDyn: towards dynamic graphics and annotations for analyses of trees.* BMC Bioinformatics. 2006, Oct 10;7:439.
x44. Howard DH. Acquisition, transport, and storage of iron by pathogenic fungi. Clin Microbiol Rev. 1999 Jul;12(3):394-404.
x45. Mateos FV, Rickauer M, Esquerré-Tugayé MT. Cloning and characterization of a cDNA encoding an elicitor of Phytophthora parasitica var. nicotianae that shows cellulose-binding and lectin-like activities. Mol Plant Microbe Interact. 1997 Dec;10(9):1045-53.
x46. Brecht S, Carruthers VB, Ferguson DJ, Giddings OK, Wang G, Jakle U, Harper JM, Sibley LD, Soldati D. The toxoplasma micronemal protein MIC4 is an adhesin composed of six conserved apple domains. J Biol Chem. 2001 Feb 9;276(6):4119-27.
x47. Kamoun S, van West P, de Jong AJ, de Groot KE, Vleeshouwers VG, Govers F. A gene encoding a protein elicitor of Phytophthora infestans is down-regulated during infection of potato. Mol Plant Microbe Interact. 1997 Jan;10(1):13-20.
x48. Panabieres, F., M. Ponchet, V. Allasia, L. Cardin, and P. Ricci. 1997. Characterization of border species among Pythiaceae: several Pythium isolates produce elicitins, typical proteins from Phytophthora spp. Mycol. Res. 101:1459–1468.
x49. Cock PJ, Antao T, Chang JT, Chapman BA, Cox CJ, Dalke A, Friedberg I, Hamelryck T, Kauff F, Wilczynski B, de Hoon MJ. Biopython: freely available Python tools for computational molecular biology and bioinformatics. Bioinformatics. 2009 Jun 1;25(11):1422-3.
x50. Gaulin E, Bottin A, Dumas B. Sterol biosynthesis in oomycete pathogens. Plant Signal Behav. 2010 Mar;5(3):258-60.
x51. Madoui MA, Bertrand-Michel J, Gaulin E, Dumas B. Sterol metabolism in the oomycete Aphanomyces euteiches, a legume root pathogen. New Phytol. 2009;183(2):291-300.
x52. Lascombe MB, Retailleau P, Ponchet M, Industri B, Blein JP, Prangé T. Structure of sylvaticin, a new alpha-elicitin-like protein from Pythium sylvaticum. Acta Crystallogr D Biol Crystallogr. 2007 Oct;63(Pt 10):1102-8.
x53. Arnold K, Bordoli L, Kopp J, Schwede T. The SWISS-MODEL workspace: a web-based environment for protein structure homology modelling. Bioinformatics 2006; 22(2):195-201.
x54. Schwede T, Kopp J, Guex N, Peitsch MC. SWISS-MODEL: An automated protein homology-modeling server. Nucleic Acids Res 2003; 31(13):3381-3385.
x55. Kiefer F, Arnold K, Kunzli M, Bordoli L, Schwede T. The SWISS-MODEL Repository and associated resources. Nucleic Acids Res 2009; 37(Database issue):D387-D392.
x56. Bordoli L, Kiefer F, Arnold K, Benkert P, Battey J, Schwede T. Protein structure homology modeling using SWISS-MODEL workspace. Nat Protoc 2009; 4(1):1-13.
x57. Pettersen EF, Goddard TD, Huang CC, Couch GS, Greenblatt DM, Meng EC et al. UCSF Chimera--a visualization system for exploratory research and analysis. J Comput Chem 2004; 25(13):1605-1612.
x58. Irwin JJ, Shoichet BK. ZINC--a free database of commercially available compounds for virtual screening. J Chem Inf Model 2005; 45(1):177-182.
x59. Kamoun, S. 2003. Molecular genetics of pathogenic oomycetes. Eukaryot. Cell. 2:191-9.
x60. Keeling, P.J., G. Burger, D.G. Durnford, B.F. Lang, R.W. Lee, R.E. Pearlman, A.J. Roger, and M.W. Gray. 2005. The tree of eukaryotes. Trends. Ecol. Evol. 20:670-6.
x61. Kwon-Chung, K.J. 1994. Phylogenetic spectrum of fungi that are pathogenic to humans. Clin. Infect. Dis. 19:S1-7.

x62. Krajaejun, T., B. Sathapatayavongs, R. Pracharktam, P. Nitiyanant, P. Leelachaikul, W. Wanachiwanawin, A. Chaiprasert, P. Assanasen, M. Saipetch, P. Mootsikapun, P. Chetchotisakd, A. Lekhakula, W. Mitarnun, S. Kalnauwakul, K. Supparatpinyo, R. Chaiwarith, S. Chiewchanvit, N. Tananuvat, S. Srisiri, C. Suankratay, W. Kulwichit, M. Wongsaisuwan, and S. Somkaew. 2006. Clinical and epidemiological analyses of human pythiosis in Thailand. Clin. Infect. Dis. 43:569-76.
x63. Mendoza, L., L. Ajello, and M.R. McGinnis. 1996. Infection caused by the Oomycetous pathogen Pythium insidiosum. J. Mycol. Med. 6:151-64.
x64. Mendoza, L., F. Hernandez, and L. Ajello. 1993. Life cycle of the human and animal oomycete pathogen Pythium insidiosum. J. Clin. Microbiol. 31:2967-73.
x65. Krajaejun T, P Chongtrakool, K Angkananukul, T T Brandhorst. 2010. Effect of temperature on growth of the pathogenic oomycete Pythium insidiosum. Southeast. Asian. J. Trop. Med. Public. Health. 41:1462-1466.
x66. Schlosser, E., and D. Gottlieb. 1966. Sterols and the sensitivity of Pythium species to filipin. J. Bacteriol. 91:1080-4.
x67. Mendoza, L., and J.C. Newton. 2005. Immunology and immunotherapy of the infections caused by Pythium insidiosum. Med. Mycol. 43:477-86.
x68. Kamoun S. A catalogue of the effector secretome of plant pathogenic oomycetes. Annu Rev Phytopathol. 2006;44:41-60.
x69. Iseli,C., Jongeneel,C.V. and Bucher,P. (1999) ESTScan: a program for detecting, evaluating, and reconstructing potential coding regions in EST sequences. Proc. Int. Conf. Intell. Syst. Mol. Biol., 7, 138–148.
x70. Wu,J., Mao,X., Cai,T., Luo,J. and Wei,L. (2006) KOBAS server: a web-based platform for automated annotation and pathway identification. Nucleic Acids Res., 34, W720–W724.
x71. Conesa,A., Gotz,S., Garcia-Gomez,J.M., Terol,J., Talon,M. and Robles,M. (2005) Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. Bioinformatics, 21, 3674–3676.
x72. Bendtsen JD, Nielsen H, von Heijne G, Brunak S. Improved prediction of signal peptides: SignalP 3.0. J Mol Biol. 2004 Jul 16;340(4):783-95.
x73. Wanachiwanawin W. Infections in E-beta thalassemia. J Pediatr Hematol Oncol. 2000 Nov-Dec;22(6):581-7.
x74. Jiang RH, Tripathy S, Govers F, Tyler BM. RXLR effector reservoir in two Phytophthora species is dominated by a single rapidly evolving superfamily with more than 700 members. Proc Natl Acad Sci U S A. 2008 Mar 25;105(12):4874-9.
x75. Dou D, Kale SD, Wang X, Jiang RH, Bruce NA, Arredondo FD, Zhang X, Tyler BM. RXLR-mediated entry of Phytophthora sojae effector Avr1b into soybean cells does not require pathogen-encoded machinery. Plant Cell. 2008 Jul;20(7):1930-47.
x76. Dou D, Kale SD, Wang X, Chen Y, Wang Q, Wang X, Jiang RH, Arredondo FD, Anderson RG, Thakur PB, McDowell JM, Wang Y, Tyler BM. Conserved C-terminal motifs required for avirulence and suppression of cell death by Phytophthora sojae effector Avr1b. Plant Cell. 2008 Apr;20(4):1118-33.
x77. Allen RL, Bittner-Eddy PD, Grenville-Briggs LJ, Meitz JC, Rehmany AP, Rose LE, Beynon JL: Host-parasite coevolutionary conflict between Arabidopsis and downy mildew. Science 2004, 306:1957-1960.
x78. Rehmany AP, Gordon A, Rose LE, Allen RL, Armstrong MR, Whisson SC, Kamoun S, Tyler BM, Birch PR, Beynon JL: Differential recognition of highly divergent downy mildew avirulence gene alleles by RPP1 resistance genes from two Arabidopsis lines. Plant Cell 2005, 17:1839-1850.
x79. Shan W, Cao M, Leung D, Tyler BM: The Avr1b locus of Phytophthora sojae encodes an elicitor and a regulator required for avirulence on soybean plants carrying resistance gene Rps1b. Mol Plant–Microbe Interact 2004, 17:394-403.
x80. Armstrong MR, Whisson SC, Pritchard L, Bos JI, Venter E, Avrova AO, Rehmany AP, Bohme U, Brooks K, Cherevach I et al.: An ancestral oomycete locus contains late blight avirulence gene Avr3a, encoding a protein that is recognized in the host cytoplasm. Proc Natl Acad Sci USA 2005,102:7766-7771.
x81. Marti M, Good RT, Rug M, Knuepfer E, Cowman AF. Targeting malaria virulence and remodeling proteins to the host erythrocyte. Science. 2004 Dec 10;306(5703):1930-3.
x82. Hiller NL, Bhattacharjee S, van Ooij C, Liolios K, Harrison T, Lopez-Estraño C, Haldar K. A host-targeting signal in virulence proteins reveals a secretome in malarial infection. Science. 2004 Dec 10;306(5703):1934-7
x83. Rug M, Prescott SW, Fernandez KM, Cooke BM, Cowman AF. The role of KAHRP domains in knob formation and cytoadherence of P falciparum-infected human erythrocytes. Blood. 2006 Jul 1;108(1):370-8.
X84. Laura Baxter, Sucheta Tripathy, Naveed Ishaque, Nico Boot, Adriana Cabral, Eric Kemen, Marco Thines, Audrey Ah-Fong, Ryan Anderson, Wole Badejoko, Peter Bittner-Eddy, Jeffrey L. Boore, Marcus C. Chibucos, Mary Coates, Paramvir Dehal, Kim Delehaunty, Suomeng Dong, Polly Downton, Bernard Dumas, Georgina Fabro, Catrina Fronick, Susan I. Fuerstenberg, Lucinda Fulton, Elodie Gaulin, Francine Govers, Linda Hughes, Sean Humphray, Rays H. Y. Jiang, Howard Judelson, Sophien Kamoun, Kim

Kyung, Harold Meijer, Patrick Minx, Paul Morris, Joanne Nelson, Vipa Phuntumart, Dinah Qutob, Anne Rehmany, Alejandra Rougon-Cardoso, Peter Ryden, Trudy Torto-Alalibo, David Studholme, Yuanchao Wang, Joe Win, Jo Wood, Sandra W. Clifton, Jane Rogers, Guido Van den Ackerveken, Jonathan D. G. Jones, John M. McDowell, Jim Beynon, and Brett M. Tyler. 2010. Signatures of Adaptation to Obligate Biotrophy in the Hyaloperonospora arabidopsidis Genome. Science. 330 (6010), 1549-1551
X85. Nürnberger T, Brunner F, Kemmerling B, Piater L. Innate immunity in plants and animals: striking similarities and obvious differences. Immunol Rev. 2004 Apr;198:249-66.

TABLES AND FIGURES

Table 1. Best BLASTX match for all 97 non-ribosomal protein-encoding genes derived from the EST dataset of *P. insidiosum*

PinsEST ID	Best BLASTX match	E-value	Species	Accession No.
001	Calmodulin	5.84E-79	*Pythium splendens*	P27165
003	Elicitin-like protein RAL8B	2.81E-16	*Phytophthora ramorum*	ABB55971.1
004	Glutaredoxin	8.48E-26	*Ricinus communis*	CAA89699.1
006	Hydrogen-transporting ATP synthase	2.51E-06	*Arabidopsis thaliana*	NP_175576.1
016	Peptidylprolyl isomerase (FKBP-type)	1.02E-39	*Tetrahymena thermophila*	XP_001014237.1
021	Putative abnormal wing disc-like protein	5.18E-56	*Maconellicoccus hirsutus*	ABM55663.1
025	Elicitin-like protein SOL7	1.48E-16	*Phytophthora sojae*	ABB56030.1
027	Carbonic anhydrase 2	5.00E-80	*Neurospora crassa*	XM_954583.2
031	CDP-alcohol phosphatidyltransferase	6.00E-06	*Spirosoma linguale*	YP_003386374.1
034	Uridine phosphorylase	1.89E-37	*Aedes aegypti*	EAT42652.1
035	Annexin	7.32E-79	*Saprolegnia monoica*	ABC59142.1
036	Elongation factor alpha-like protein	2.06E-83	*Chlamydomonas incerta*	ABA01120.1
037	Wheat adenosylhomocysteinase-like protein	1.00E-56	*Oryza sativa*	AAO72664.1
038	Glutamine amidotransferase	7.16E-26	*Bradyrhizobium sp.*	ZP_00857064.1
039	Mitochondrial carrier protein	2.18E-59	*Trypanosoma brucei*	XP_827321.1
042	Elicitin-like protein 5 precursor	8.80E-05	*Phytophthora phaseoli*	ABG91746.1
044	Qm CG17521-PB, isoform B	2.98E-85	*Drosophila melanogaster*	NP_730773.3
045	Elicitin-like protein RAL7A	4.72E-18	*Phytophthora ramorum*	ABB55968.1
046	Elicitin-like protein SOL7	4.02E-16	*Phytophthora sojae*	ABB56030.1
052	Similar to Os09g0542200	1.30E-28	*Strongylocentrotus purpuratus*	XP_790435.1
054	Actin-depolymerizing factor	7.92E-25	*Platanus x acerifolia*	CAL25339.1
055	Superoxide dismutase	2.86E-38	*Aplysia californica*	AAM44291.1
056	V-type atpase 16 kda proteolipid subunit	2.52E-45	*Pleurochrysis carterae*	Q43362
059	Endopeptidase/ peptidase/ threonine endopeptidase	2.91E-46	*Arabidopsis thaliana*	NP_191641.1
066	Elicitin-like protein BRL1B	4.39E-09	*Phytophthora brassicae*	AAO92427.1
067	Thioredoxin-like protein	1.59E-08	*Bombyx mori*	ABJ97191.1
068	COX17 cytochrome c oxidase assembly homolog	2.16E-13	*Apis mellifera*	XP_001122739.1
069	Succinate-coa ligase	5.94E-32	*Dictyostelium discoideum*	XP_645537.1
070	Mitochondrial import inner membrane translocase subunits	1.23E-08	*Aedes aegypti*	ABF18336.1
071	V-atpase subunit c" proteolipid	4.75E-38	*Xerophyta viscosa*	AAR26002.1
073	Acyl-CoA-binding protein	5.69E-14	*Photobacterium profundum*	YP_130412.1
074	Catechol O-methyltransferase	4.85E-37	*Bos taurus*	XP_875703.2
077	Peptidylprolyl isomerase (cyclophilin-type)	2.00E-77	*Phytophthora infestans*	AAN31483.1
078	Macrophage migration inhibitory factor	3.18E-17	*Maconellicoccus hirsutus*	ABM55630.1
082	Copine	5.49E-46	*Entamoeba histolytica*	XP_656751.1
086	Hypothetical protein	5.64E-06	*Magnaporthe grisea*	XP_364019.1
087	Hypothetical protein	7.40E-06	*Ostreococcus tauri*	CAL51758.1
089	Ubiquinol--cytochrome c reductase	1.32E-10	*Solanum tuberosum*	P48504
091	C-Myc-binding protein	6.00E-15	*Perkinsus marinus*	EER12924.1
096	Cytochrome c	4.50E-35	*Spinacia oleracea*	P00073
097	Transmembrane protein 93	6.00E-14	*Saccoglossus kowalevskii*	XP_002739344.1
099	Ankyrin repeat-containing protein	6.00E-17	*Zygosaccharomyces rouxii*	CAQ43291.1

100	Hypothetical protein	1.75E-05	*Plasmodium falciparum*	XP_001349024.1
103	Maleylacetoacetate isomerase	8.26E-62	*Dictyostelium discoideum*	XP_642170.1
104	Fatty acid elongase	1.90E-39	*Trypanosoma cruzi*	XP_818064.1
106	Acyl-CoA independent ceramide synthase	3.86E-29	*Arabidopsis thaliana*	NP_567660.1
108	Eukaryotic translation initiation factor 5A	9.91E-47	*Spodoptera frugiperda*	P62924
112	CBEL protein	1.02E-09	*Phytophthora parasitica*	CAA65843.1
114	Nucleoside phosphorylase	8.48E-45	*Danio rerio*	NP_998476.1
115	Mitochondrial ATP synthase gamma-subunit	7.85E-17	*Acyrthosiphon pisum*	ABD91518.1
116	Branched-chain-amino-acid aminotransferase	1.00E-66	*Phytophthora infestans*	EEY63223.1
118	Small nuclear riboprotein Sm-D1	4.71E-38	*Arabidopsis thaliana*	NP_192193.1
119	Hypothetical protein	1.12E-14	*Phytophthora infestans*	AAY43427.1
120	Small ribonucleoprotein D2-like protein	1.05E-37	*Chlamys farreri*	AAM94277.1
121	Thioredoxin-like protein	8.62E-26	*Gallus gallus*	XP_424463.1
124	Ubiquitin ubia	4.71E-60	*Aspergillus fumigatus*	XP_750152.1
129	Nucleolar protein 16	1.00E-06	*Homo sapiens*	NP_057475.2
131	Eukaryotic translation initiation factor 6	1.82E-82	*Bombyx mori*	NP_001040517.1
135	3-hydroxybutyryl-coa dehydrogenase	1.93E-25	*Acidobacteria bacterium*	YP_590288.1
136	Phosphoglycerate mutase	2.06E-73	*Dictyostelium discoideum*	XP_638289.1
138	FYVE-type zinc finger-containing protein	8.00E-20	*Polysphondylium pallidum*	EFA78347.1
140	RNA polymerase III, subunit C34	2.50E-31	*Ostreococcus tauri*	CAL58583.1
141	S-adenosyl methionine synthetase	1.72E-117	*Phytophthora infestans*	AAN31489.1
142	Hypothetical protein	7.86E-06	*Tetrahymena thermophila*	XP_001022125.1
146	Polyunsaturated fatty acid elongase 1	7.91E-58	*Thalassiosira pseudonana*	AAV67799.1
147	Retrotransposon protein	1.00E-04	*Oryza sativa Indica Group*	ABR26094.1
148	Ubiquitin-like protein	3.51E-16	*Arabidopsis thaliana*	CAA67923.1
149	Hypothetical protein	3.42E-27	*Phytophthora infestans*	AAN31498.1
150	Hypothetical protein	3.00E-06	*Chlamydomonas reinhardtii*	XP_001701958.1
151	Hypothetical protein	1.82E-08	*Aspergillus terreus*	XP_001211584.1
152	Hypothetical protein	3.84E-10	*Arabidopsis thaliana*	NP_566572.1
156	Rotamase	7.69E-33	*Aedes aegypti*	EAT38181.1
158	Nucleoredoxin	7.09E-25	*Homo sapiens*	EAW90639.1
160	Ferrochelatase	2.58E-96	*Bos taurus*	NP_776479.1
161	Small GTP binding protein Rab2A	3.82E-88	*Saccharum officinarum*	ABD59354.1
162	Cyclopropane-fatty-acyl-phospholipid synthase family protein	1.27E-14	*Synechococcus sp.*	YP_475654.1
165	Histidine triad protein	3.69E-40	*Medicago truncatula*	ABE83728.1
166	Heat shock transcription factor 2	1.82E-13	*Gallus gallus*	XP_419760.2
168	Putative endonuclease	5.92E-26	*Myxococcus xanthus*	YP_630638.1
173	Similar to Lsm3 protein	3.93E-31	*Gallus gallus*	XP_414380.1
174	Histone H2A	4.51E-11	*Saccharomyces cerevisiae*	NP_010429.1
177	Aspartyl aminopeptidase	3.38E-79	*Neosartorya fischeri*	XP_001263609.1
179	RNA polymerase II core subunit	3.88E-30	*Dictyostelium discoideum*	XP_642084.1
182	Small cysteine rich protein SCR76	4.11E-33	*Phytophthora infestans*	AAN31495.1
184	Translocase of inner mitochondrial membrane 13 homolog	3.40E-11	*Tribolium castaneum*	XP_972097.1
186	Translation factor	1.41E-129	*Medicago truncatula*	ABE83172.1
191	Histone H4	5.87E-39	*Ostreococcus tauri*	CAL50535.1
194	Actin	6.65E-180	*Pythium splendens*	AAG01044.1
197	Peptidylprolyl isomerase (FKBP-type)	4.03E-46	*Vicia faba*	O04287
200	Chaperonin 10	1.57E-19	*Brassica napus*	Q96539
202	Methionine sulfoxide reductase	1.21E-46	*Plantago major*	CAJ34818.1
203	Transmembrane protein c20orf108	1.00E-07	*Ixodes scapularis*	XP_002407904.1
205	Rac GTPase activator	3.86E-06	*Arabidopsis thaliana*	NP_187756.1
208	Cytoplasmic membrane protein	6.00E-09	*Leptospira interrogans*	YP_003621.1
209	Nascent polypeptide-associated complex alpha polypeptide	2.70E-08	*Oreochromis niloticus*	Q8AWF2
215	Aminoacyl-trna synthetase	3.16E-21	*Medicago truncatula*	ABE91897.1
216	TB2/DP1/HVA22 family integral membrane protein	1.10E-31	*Cryptosporidium parvum*	XP_628022.1

Table 2: Utilization of InterProScan [x32] for identification of protein domains of *P. insidiosum*'s predicted gene products those either matched to hypothetical proteins of other organisms (as indicated by "*") or had no BLASTX hit.

PinsEST ID	Protein domain	InterProScan Accession no.	E-value
002	B12D	IPR010530	7.50E-05
007	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 4	IPR019550	7.00E-06
024	Cytochrome c oxidase, subunit Via	IPR001349	8.20E-06
030	Armadillo	IPR000225	2.30E-06
149*	B-cell receptor-associated 31-like	IPR008417	7.60E-07
050	MARVEL-like domain	IPR021128	5.30E-14
152*	Complex 1 LYR protein	IPR008011	6.50E-07
076	Ribosomal protein 60S	IPR001813	7.10E-10
102	Ubiquinol-cytochrome C reductase, UQCRX/QCR9-like	IPR008027	2.20E-15
113	Phox-like	IPR001683	1.40E-10
127	Lipid-binding START	IPR002913	8.70E-15
143	Mitochondrial outer membrane translocase complex, subunit Tom7	IPR012621	6.30E-09
170	Uncharacterised protein family UPF0197	IPR007915	2.80E-12
176	Elicitin	IPR002200	8.50E-06
196	Cytochrome c oxidase, subunit Vb	IPR002124	1.30E-10

Table 3: Unigenes of *P. insidiosum* that are highly represented (≥ 4 clones) in the EST dataset. "*" indicates the *P. insidiosum* genes those are predicted to contain signal peptide by the SignalP 3.0 program (x04).

PinsEST ID	Number of clones	BLASTX hit
014*	37	--- no hit ---
003*	20	Elicitin-like protein RAL8B
004	20	Glutaredoxin
013	9	Ribosomal protein S21e
028	9	Ribosomal protein L9
001	8	Calmodulin
008	8	--- no hit ---
061	8	Ribosomal protein S11
009	7	--- no hit ---
017	7	Ribosomal protein L23
025*	7	Elicitin-like protein SOL7
045*	7	Elicitin-like protein RAL7A
050	7	--- no hit ---
022	6	Ribosomal protein L36
023	6	Ribosomal protein L37
047	6	Ribosomal protein S15e
057	6	Ribosomal protein L39
063	6	Ribosomal protein S27
006	5	Hydrogen-transporting ATP synthase
053	5	--- no hit ---
072*	5	--- no hit ---
078	5	Macrophage migration inhibitory factor
016	4	Peptidylprolyl isomerase (FKBP-type)
024	4	--- no hit ---
026*	4	--- no hit --- ---
033	4	--- no hit ---
035	4	Annexin
051	4	Ribosomal protein L7
056	4	V-type ATPase
080	4	Ribosomal protein L27e

Table 4: Identification of putative pathogenicity genes of *P. insidiosum* by literature review of the previously-reported pathogenicity genes of other pathogens.

PinsEST ID	Matched pathogenicity genes of other pathogens						Function	Ref
	Gene	Gene product	E-value[a]	Accession no.[b]	Pathogen	Host		
001	*CAM1*	Calmodulin	2.00E-71	XP_569886	*Cryptococcus neoformans*	Human	Body temperature growth	x08
055	*SOD1*	Cu-Zn superoxide dismutase	8.00E-38	AAK31918.1	*Cryptococcus neoformans*	Human	Antioxidant and virulence	x10
160	*hemH*	Ferrochelatase	4.00E-29	AY027659	*Brucella abortus*	Human	Heme metabolism and virulence	x12
027	*NCE103*	β-carbonic anhydrase	1.00E-31	AACQ01000013.1	*Candida albicans*	Human	Growth in CO_2-limited condition	x09
166	*Hsf1*	Heat shock transcription factor	3.00E-14	orf19.4775	*Candida albicans*	Human	Thermal adaptation and virulence	x13
004	*CaGRX2*	Glutaredoxin	3.00E-25	orf19.6059	*Candida albicans*	Human	Antioxidant and virulence	x11
078	*PfMIF*	Macrophage migration inhibitory factor	7.00E-15	AY561832.1	*Plasmodium falciparum*	Human	Immunomodulator	x14
067	*Trx1*	Thioredoxin	3.00E-16	orf19.7611	*Candida albicans*	Human	Antioxidant and virulence	x15
112	*CBEL1*	Cellulose binding elicitor lectin (CBEL)	1.00E-12	X97205.1	*Phytophthora parasitica*	Plant	Effector, adhesion and hemagglutination	x45
003[c]	*INF1*	Elicitin	2.00E-13	AY830090.1	*Phytophthora infestans*	Plant	Effector	x17, x47

Footnote:

[a] Accession numbers of the *C. albicans* genes for the *C. albicans* genome database (http://www.candidagenome.org/cgi-bin/seqTools).

[b] E-value from two-sequence BLASTP analysis of putative pathogenicity gene of *P. insidiosum* and that of pathogenicity gene from other pathogens.

[c] Encoded protein of PinsEST# was assigned to be INSL1. There are six other *P. insidosum* elicitin-like proteins [INSL2 (PinsEST#025), INSL3 (PinsEST#042), INSL4 (PinsEST#045), INSL5 (PinsEST#046), INSL6 (PinsEST#066) and INSL7 (PinsEST#176)] with E-value ranging from 3.00E-06 to 8.00E-10, when compared to the elicitin INF1 of *Phytophthora infestans.*

Figure 1: Characteristics of the P. insidiosum EST dataset: (A) Length distribution of all 486 EST; and (B) Redundancy of all 217 *P. insidiosum* unigenes, as indicated by number of clone(s) per unigene.

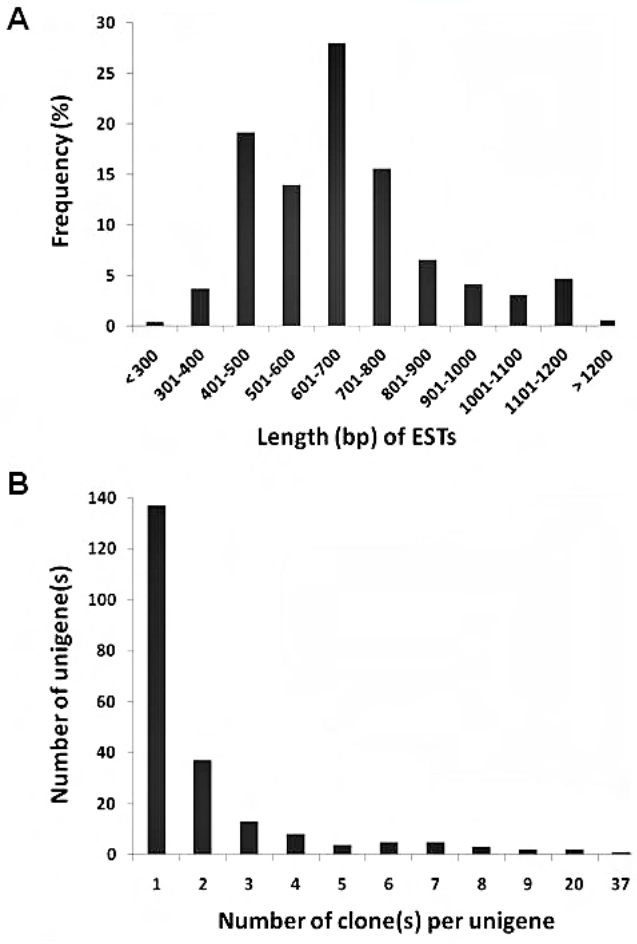


Figure 2 Protein domain organization of some representative effectors from oomycetes and pathogenic parasites. Schematic structures showing functional domains of effecotrs include that of (i) elicitins (INF1 from *P. infestans*, RAL8A from *P. ramorum*, and INSL1 (PinsEST#003), INSL2 (PinsEST#025) and INSL7 (PinEST#176) from *P. insidiosum*), (iii) RXLR-dEER domain or Plasmodium export element (PEXEL; containing RXLX motif)-containing molecules (Avr1b from *P. sojae*, ATR13 from *H. parasitica*, the knob-associated histidine-rich protein (KAHRP) from the malaria parasite *P. falciparum*, and INSL7 (PinEST#176) from *P. insidiosum*), and (iii) PAN/Apple domain-containing proteins (CBEL for *P. parasitica*, MIC4 from *T. gondii*, and PinsPAN1 (PinsEST#112) from *P. insidiosum*). All structures were depicted in relative proportion, with the ruler on the top to indicate length (in amino acids) of each protein. Abbreviations: SP, signal peptide; R, the RXLR domain or PEXEL; W and Y, conserved domains in C-terminus of the RXLR effectors of *Pytophthora* species [x74]; CBD, cellulose binding domain; PAN/Apple, the PAN/Apple domain.

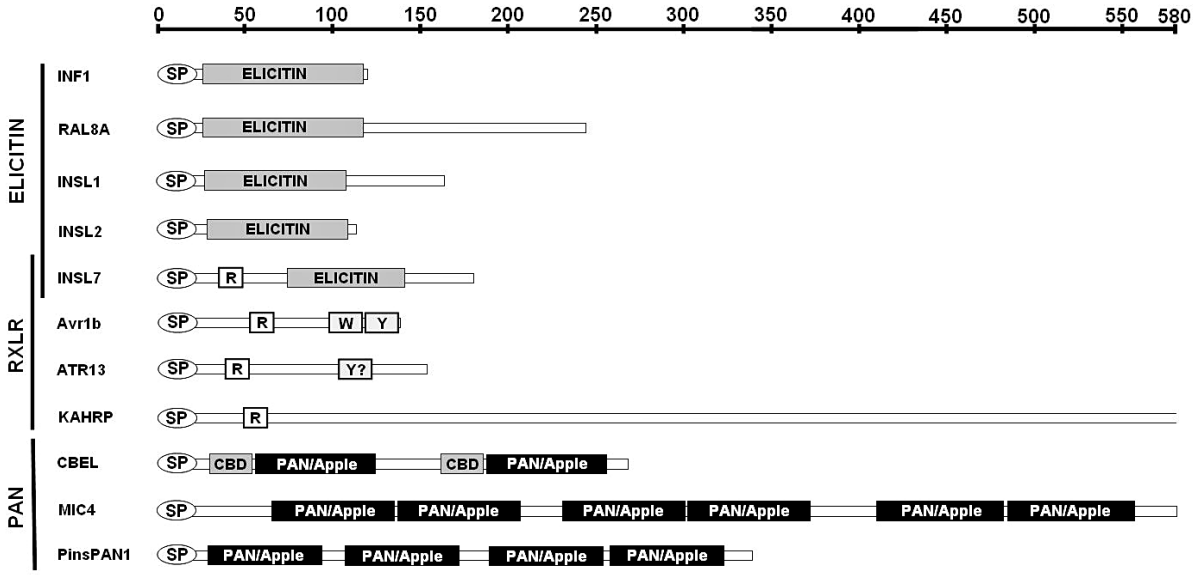


Figure 3: Multiple sequence alignment of the RXLR-dEER domains of a putative RXLR effector from *P. insidiosum* (INSL7) and that of four prototypic RXLR effectors from other pathogenic oomycetes: ATR1 and ATR13 from *H. parasitica*, Avr1b from *P. sojae* and Avr3a from *P.infestans*. The box indicates the highly-conserved RXLR motif. The underlines indicate acidic residues (D/E) of the dEER motif.

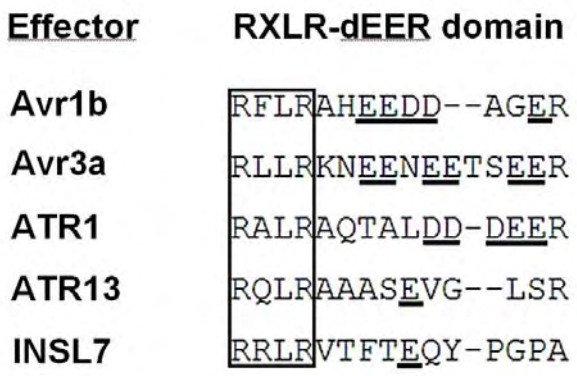


Figure 4. Multiple protein sequence alignment of the elicitin domains of all identified *P. insidiosum* elicitin-like proteins (INSL1-7) and some elicitins from other oomycetes: *Phytophthora cryptogea* (CRY), *Pythium vexans* (VEX1), *Phytophthora sojae* (SOJB), *Phytophthora brassicae* (BRA2), *Phytophthora ramorum* (RAM3B), and *Phytophthora infestans* (INF5). The 6 conserved cysteine residues were marked with C1, C2, C3, C4, C5 and C6. Based on the defined structure of CRY, these cysteine residues form 3 pairs of disulfide bonds: C1-C5, C2-C4 and C3-C6). "*" indicates ergosterol interactive sites of CRY [x37].

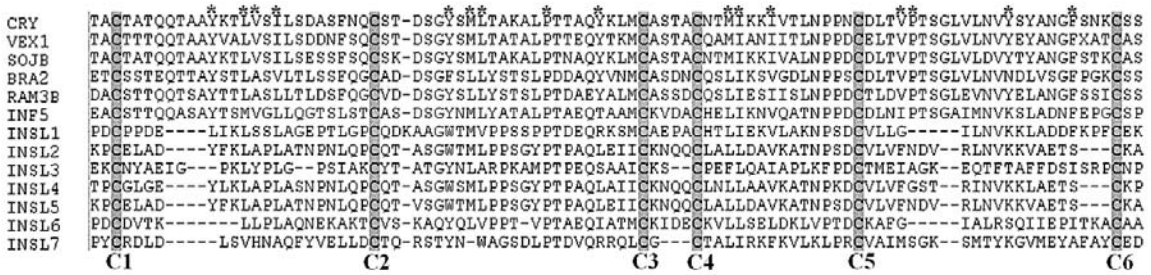


Figure 5. An unrooted phylogenetic tree of 45 elicitin domain sequences from *Pythium insidiosum* (INSL1-7; n=7), *Pythium oligandrum* (OLI; n=1), *Pythium vexans* (VEX1 and VEX2; n=2), *Phytophthora cryptogea* (CRY; n=1), *Phytophthora brassicae* (BRL1B, BRA2, and BRA6; n=3), *Phytophthora infestans* (INF1, INF2A, INF5, INL1, INL2, INL3A, INL4B, INL11B and INL13; n=9), *Phytophthora ramorum* (RAM3B, RAL2A, RAL5, RAL6, RAL7B, RAL8A, RAL9, RAL10A, RAL10B, RAL11A, RAL12, and RAL13A; n=12), and *Phytophthora sojae* (SOJ1E, SOJ3B, SOL3A, SOL4B, SOL5, SOL6, SOL7, SOL8, SOL9 and SOL12; n=10), using Neighbor-Joining analysis. The reliability of the inferred trees was tested using bootstrap re-sampling with 1,000 replicates (indicated by the percentage of replicates supporting each branch). Amino acid sequences of ELIs and ELLs used in this analysis are obtained from the report of Rays et. al. [x17].

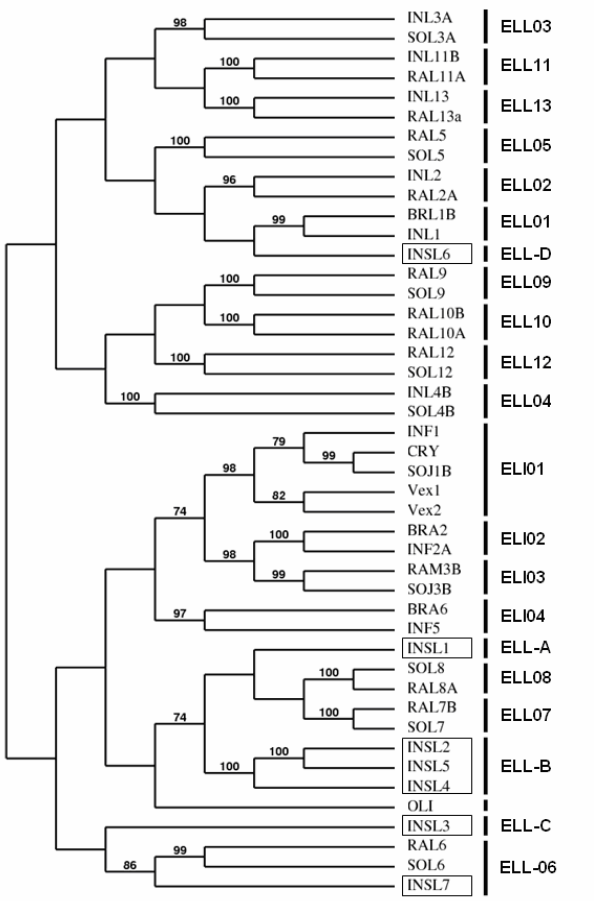
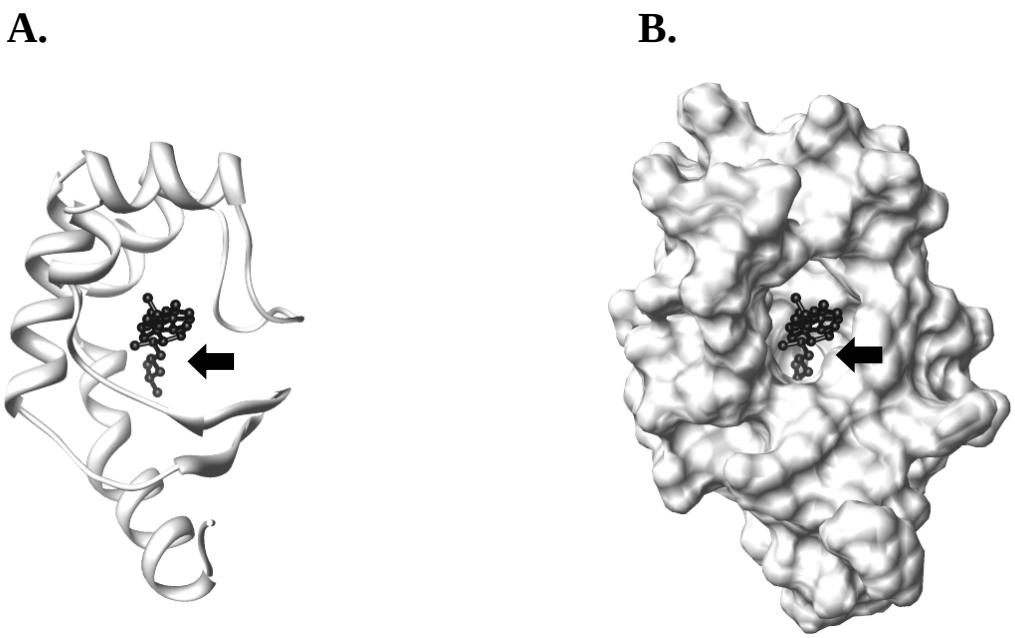


Figure 6. Predicted three-dimentional structures of the putative elicitin-like INSL2 (PinsEST#025) of *P. insidiosum* in complex with one molecule of cholesterol (shown as ball and stick model, indicated by an arrow), which is encapsulated in the pocket-like cavity of the INSL2 elicitin: (A) Ribbon structure model, and (B) Surface structure model of INSL2. See the methods for details of the construction and docking analyses.



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The 74-Kilodalton Immunodominant Antigen of the Pathogenic Oomycete *Pythium insidiosum* Is a Putative Exo-1,3-ß-Glucanase▽

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The oomycetous, fungus-like, aquatic organism *Pythium insidiosum* is the causative agent of pythiosis, a life-threatening infectious disease of humans and animals living in tropical and subtropical areas of the world. Common sites of infection are the arteries, eyes, cutaneous/subcutaneous tissues, and gastrointestinal tract. Diagnosis of pythiosis is time-consuming and difficult. Radical excision of the infected organs is the main treatment for pythiosis because conventional antifungal drugs are ineffective. An immunotherapeutic vaccine prepared from *P. insidiosum* crude extract showed limited efficacy in the treatment of pythiosis patients. Many pythiosis patients suffer lifelong disabilities or die from an advanced infection. Recently, we identified a 74-kDa major immunodominant antigen of *P. insidiosum* which could be a target for development of a more effective serodiagnostic test and vaccines. Mass spectrometric analysis identified two peptides of the 74-kDa antigen (s74-1 and s74-2) which perfectly matched a putative exo-1,3-ß-glucanase (EXO1) of *Phytophthora infestans*. Using degenerate primers derived from these peptides, a 1.1-kb product was produced by PCR, and its sequence was found to be homologous to that of the *P. infestans* exo-1,3-ß-glucanase gene, *EXO1*. Enzyme-linked immunosorbent assays targeting the s74-1 and s74-2 synthetic peptides demonstrated that the 74-kDa antigen was highly immunoreactive with pythiosis sera but not with control sera. Phylogenetic analysis using part of the 74-kDa protein-coding sequence divided 22 Thai isolates of *P. insidiosum* into two clades. Further characterization of the putative *P. insidiosum* glucanase could lead to new diagnostic tests and to antimicrobial agents and vaccines for the prevention and management of the serious and life-threatening disease of pythiosis.

Oomycetes are a unique group of fungus-like eukaryotic microorganisms that belong to the stramenopiles of the supergroup Chromalveolates and are biochemically and genetically different from true fungi (13, 14, 20). Phylogenetic analysis showed that the oomycetes are more closely related to diatoms and algae (20). Many of the oomycetes are plant pathogens that cause significant agricultural losses each year, while some are capable of infecting humans and animals, including horses, dogs, cats, cattle, sheep, fish, and mosquitoes (13). Finding a way to control the infections caused by the oomycetes is challenging.

Pythium insidiosum is the only oomycete that causes life-threatening infectious disease in humans and animals (mostly horses and dogs). The disease state is called pythiosis, and it is endemic to tropical and subtropical regions (13, 19, 24). In nature, *P. insidiosum* inhabits swampy areas, where it is present in one of two forms: a right-angle branching mycelium and a biflagellate zoospore (25). The zoospore is a motile asexual morphotype that can germinate to produce mycelia on its natural hosts, such as water plants (25). Infection occurs when zoospores come in contact with humans or animals and invade as germinating hyphae (25). In humans, the most common sites of infection are arteries and eyes (19), while in animals, cutaneous/subcutaneous and gastrointestinal sites predominate (24).

High rates of morbidity and mortality of pythiosis are exacerbated by the lack of early diagnosis and effective treatments (19, 24). Diagnosis by culture identification is time-consuming and limited to experienced clinical laboratories (6, 29). Serological and PCR-based assays have been developed for facilitating the diagnosis of pythiosis (4, 9, 10, 12, 15–18, 26, 27, 34, 39, 42, 43). The PCR-based diagnostic procedure is complicated, and the necessary materials and equipment are not widely available. All of the reported serology-based assays require a crude extract of *P. insidiosum* as the antigen source. In our experience, batch-to-batch variation in the quality of the crude antigen leads to problems with the reproducibility of the serological assays. For each new antigen preparation, the assay protocol needs extensive reevaluation in regards to antigen concentration, the optimal dilution of the serum samples, cutoff points, and the effective concentration of other reagents before it is suitable for clinical use. In addition, recent reports

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TABLE 1. *P. insidiosum* isolates from Thai patients with pythiosis used in this study

Clinical isolate	Geographic origin (province)	Form of infection	Reference	GenBank accession no.[a]
P1	Bangkok	Artery	Personal collection	GU994090
P2	Lopburi	Artery	Personal collection	GU994091
P3	Chantaburi	Artery	Personal collection	GU994092
P6	Nan	Artery	CBS119452	GU994093
P7	Ayuthaya	Artery	Personal collection	GU994094
P9	Lumpang	Artery	CBS119453	GU994095
P10	NA[b]	Cut	CBS673.85	GU994096
P11	Saraburi	Cut	Personal collection	GU994097
P15	Yasothon	Cornea	Personal collection	GU994098
P16	Saraburi	GI[c]	Personal collection	GU994099
P17	Chonburi	Cornea	Personal collection	GU994100
P19	NA	Cornea	Personal collection	GU994101
P20	NA	Cornea	Personal collection	GU994102
P22	Patomthani	Cornea	Personal collection	GU994103
P24	Supanburi	Artery	Personal collection	GU994104
P25	Nakornpatom	Artery	Personal collection	GU994105
P27	NA	Cornea	Personal collection	GU994106
P28	NA	Artery	Personal collection	GU994107
P29	NA	Artery	Personal collection	GU994108
P30	Rachaburi	Artery	Personal collection	GU994109
P32	NA	Cornea	Personal collection	GU994110
P40	Pichit	Artery	Personal collection	GU994111

[a] Accession number of the putative exo-1,3-ß-glucanase partial gene sequence of each *P. insidiosum* isolate used in this study.
[b] NA, not available.
[c] GI, gastrointestinal tract.

of false-positive results for some diagnostic tests (12, 15) indicate a problem regarding the specificity of detection of the crude extract antigen. Taken together, these complications indicate an ongoing need for a more reliable and specific antigen source for the development of serological assays.

Use of conventional antifungal agents for treatment of pythiosis is ineffective because the oomycetes lack the drug-targeted ergosterol biosynthesis pathway (19, 38). Surgical removal of the affected organs (i.e., the eye or leg) is the main treatment to control the disease, but it leads to lifelong physical handicaps (19, 24). Despite intensive care, many pythiosis patients die from an advanced and aggressive infection. An immunotherapeutic vaccine prepared from crude extracts of *P. insidiosum* showed curative rates of up to 60% for horses, 56% for humans, and 33% for dogs with pythiosis (19, 28, 44). Improved outcomes can result from early diagnosis and effective treatment, so these are still important health care goals.

A major immunodominant antigen of *P. insidiosum* could be a target for development of a more reliable serodiagnostic test, as well as a more effective vaccine, for patients with pythiosis. We recently identified 74- and 34- to 43-kDa major immunodominant antigens in the extracts of *P. insidiosum* human isolates by Western blot analysis (18). Only the 74-kDa immunodominant antigen was found in all *P. insidiosum* isolates and recognized by all pythiosis sera tested. Control sera from healthy individuals, patients with thalassemia (a major predisposing factor of pythiosis), and patients with various other infectious diseases failed to react with this 74-kDa immunogen (18), suggesting that this protein is specifically recognized by anti-*P. insidiosum* antibodies. The Western blot profiles of *P. insidiosum* extracts (probed with anti-*P. insidiosum* antibodies) were compared to those of *Pythium aphanidermatum* and *Pythium deliense* (which are phylogenetically related to *P. insidiosum* but which are not pathogenic for humans or animals), as well as those of *Conidiobolus coronatus* and *Rhizopus* spp. (which are zygomycetic fungi that share microscopic features with *P. insidiosum*) (18). The 74- and 34- to 43-kDa immunogens were absent in the Western blot profiles of the nonpathogenic *Pythium* spp. and zygomycetic fungi tested, suggesting that these immunogens are unique to *P. insidiosum*. Identification and characterization of these immunogenic proteins and their genes are the first step to explore their functional roles and to develop downstream clinical applications. Here, we report on our use of mass spectrometric (MS) and molecular genetic analyses to identify and characterize the proteins and their encoding genes corresponding to the 74- and 34- to 43-kDa major immunodominant antigens of *P. insidiosum*.

MATERIALS AND METHODS

Microorganisms and antigen preparations. The 22 clinical isolates of *Pythium insidiosum* used in this study and clinical details, including the form of pythiosis and the geographic origins of the patients for each isolate, are listed in Table 1. The method of antigen preparation was that described previously (18). Briefly, *P. insidiosum* strain P1 was cultured on Sabouraud dextrose agar for 3 days at 37°C. Ten small agar pieces, containing hyphal elements, from the growing culture were transferred to 100 ml of Sabouraud dextrose broth and shaken (150 rpm) for 10 days at 37°C. Merthiolate (0.02% [wt/vol]) was added to kill the cultures before they were filtered through a Durapore membrane filter (0.22-μm pore size; Millipore, County Cork, Ireland). The filtered broth containing phenylmethylsulfonyl fluoride (PMSF; 0.1 mg/ml) and EDTA (0.3 mg/ml) is referred to as culture filtrate antigen (CFA). The retained hyphal mass was transferred to a mortar in the presence of 25 ml of cold sterile distilled water containing PMSF (0.1 mg/ml) and EDTA (0.3 mg/ml). The hyphal mass was ground on ice and centrifuged at 4,800 × *g* for 10 min at 4°C. The resulting supernatant, referred to as soluble antigen from broken hyphae (SABH), was filtered through a Durapore membrane filter (0.22-μm pore). SABH and CFA were concentrated ~80-fold using an Amicon Ultra 15 centrifugal filter (10,000 nominal-molecular-weight limit; Millipore, Bedford, MA). The concentrated preparations were stored at −20°C until use.

Serum samples. Sera from five patients with vascular pythiosis diagnosed by culture identification and serological tests (serum samples T1 to T5) and five healthy blood donors (serum samples C1 to C5) who came to the Blood Bank Division, Ramathibodi Hospital, were used for evaluating the immunoreactivities of the synthetic peptides in this study. All sera were stored at −20°C until use.

SDS-PAGE and Western blotting. Sodium dodecyl sulfate (SDS)-PAGE and Western blotting analysis were performed according to the procedures described previously (18). Briefly, ~15 μg of SABH or CFA was mixed with loading buffer (60 mM Tris-HCl, pH 6.8, 25% glycerol, 2% SDS, 14.4 mM mercaptoethanol, 0.1% bromophenol blue), boiled for 5 min, and centrifuged at 14,000 × *g* for 5 min. The proteins in the supernatant were separated at 100 V in an SDS-polyacrylamide gel (4% stacking gel, 12% separating gel) on a minigel apparatus (Bio-Rad, Hercules, CA). The gel was stained with Coomassie brilliant blue. Prestained SDS-PAGE molecular weight standards (Bio-Rad) were run in parallel.

For Western blot analysis, the separated antigens were electrotransferred from SDS-polyacrylamide gels to polyvinylidene difluoride membranes (Bio-Rad) for 1 h at 100 V. The membranes were blocked with 5% gelatin in Tris-buffered saline (TBS), pH 7.5, and incubated overnight at room temperature with pythiosis serum sample T1 diluted 1:1,000 with antibody buffer (Tris-buffered saline [TBS], pH 7.5, 0.05% Tween 20, 1% gelatin). The membranes were washed twice with washing buffer (TBS, pH 7.5, 0.05% Tween 20) and incubated at room temperature for 2 h with goat anti-human immunoglobulin G (heavy plus light chains) conjugated with horseradish peroxidase (Bio-Rad) at a 1:3,000 dilution with antibody buffer. The membranes were washed with washing buffer three times. Signals were developed by adding a fresh mixture of 10 ml of 0.3% 4-chloro-1-naphthol in methanol and 50 ml of TBS with 30 μl of 30% H_2O_2. The reactions were stopped by immersing the membranes in distilled water. Membranes in which the color developed were immediately photographed.

Mass spectrometric analysis. SABH and CFA protein bands corresponding to the 43- and 74-kDa immunodominant antigens, separated by one-dimension SDS-PAGE, were excised from a Coomassie brilliant blue-stained gel and cut into 1-mm pieces. Destaining, trypsin in-gel digestion, extraction, and peptide purification were performed using the protocol of Saveliev et al. (37). The peptides were subjected to analysis on a MDS SCIEX 4800 matrix-assisted laser desorption ionization–tandem time of flight (MALDI-TOF/TOF) mass spectrometer (Applied Biosystems, Foster City, CA) at the Biotechnology Center, University of Wisconsin—Madison. Peptide mass fingerprints (PMFs) were generated and used to match theoretical trypsin-digested proteins in the National Center for Biotechnology Information (NCBI) nonredundant database using the program MASCOT (37). Some peptide masses for generating *de novo* amino acid sequence tags by MALDI-TOF/TOF tandem MS/MS analysis were selected. The *de novo* amino acid sequence of each peptide was compared with the sequences in the NCBI nonredundant protein database (blastp) by BLAST analysis.

Genomic DNA extraction, PCR, and DNA sequencing. Genomic DNA extraction was performed using the protocol of Pannanusorn et al. (31) or Vanittanakom et al. (43). The 1.1-kb region of the *P. insidiosum* ß-glucanase gene sequence was amplified by PCR using degenerate primers Pr1 (5′-ACNTTYGARCAYT GGCCNAT-3′) and Pr4 (5′-ARRTGCCARTAYTGRTTRAA-3′). Alternatively, primers Pr7 (5′-ACGTTCGAGCACTGGCCTAT-3′) and Pr10 (5′-AGG TGCCAGTACTGGTTGAA-3′) (which are subset primers of Pr1 and Pr4, respectively, that were derived from the sequence of the putative exo-1,3-ß-glucanase gene, *EXO1*, of *Phytophthora infestans*) were used to amplify the glucanase DNA sequences of some *P. insidiosum* isolates. The amplifications were carried out in a 50-μl reaction mixture containing 200 ng genomic DNA, 0.2 mM deoxynucleoside triphosphates, 1 μM each primer, and 1 μl of Elongase DNA polymerase mixture (Invitrogen) in 1× Elongase buffer (ratio of buffer A/buffer B = 1:4). Amplifications were performed in a MyCycler thermal cycler (Bio-Rad) with the following cycling temperatures: denaturation at 94°C for 2.5 min for the first cycle and 30 s for subsequent cycles, annealing for 30 s at 40°C, and elongation for 1.5 min at 68°C for a total of 35 cycles, followed by a final extension for 10 min at 68°C. The PCR products were assessed for amount and size by 1% agarose gel electrophoresis.

Direct sequencing of PCR products was performed using the BigDye Terminator (version 3.1) cycle sequencing kit (Applied Biosystems). Primers Pr1, Pr7, and Pr46 (5′-ACCTCATGTCCAAGAAGCTCAACG-3′) were used to obtain the forward sequences; and primers Pr4, Pr10, and Pr43 (5′-CGCGCATAAAG TCGAGCCAGAA-3′) were used for the reverse sequence. Automated sequencing was carried out using an ABI 3100 genetic analyzer (Applied Biosystems), and the sequences were analyzed using the Applied Biosystems sequencing software.

Synthetic peptides and ELISA. Synthetic peptides s74-1 (WTSIASTQPVGT TTFEHWPIR) and s74-2 (FLTLEEQCDWAFNQYWHLNR) (≥95% pure) were purchased from BioSynthesis Inc. (Lewisville, TX). Each peptide was dissolved in sterile distilled water. The s74-1 and s74-2 peptides were diluted to a final concentration of 0.1 μg/ml in 50 mM phosphate buffer with 0.6 M NaCl (pH 5.6) or 50 mM acetate buffer with 0.6 M NaCl (pH 2.6). Each peptide was used to coat a 96-well polystyrene plate (100 μl/well; Costar; Corning Incorporated, Corning, NY) at 4°C overnight. Unbound antigens were removed by four washes with washing buffer (0.1 M Tris-NaCl buffer with 0.1% Tween). Serum samples from pythiosis patients and blood donors were diluted 200-fold in 0.15 M phosphate-buffered saline (PBS; pH 7.2) with 1% bovine serum albumin (BSA) (PBS-BSA) and added to wells in triplicate (100 μl/well), and the plates were incubated at 37°C for 30 min and washed four times. Peroxidase-conjugated goat anti-human IgG Fcγ antibody (AffiniPure; Jackson ImmunoResearch Laboratories Inc.) was diluted 100,000-fold in PBS-BSA and added to each well (100 μl/well), and the plates were incubated at 37°C for 30 min. After unbound conjugate antibody was washed four times, 100 μl of tetramethylbenzidine solution (galactomannan enzyme-linked immunosorbent assay [ELISA] kit; Bio-Rad) was added to each well. The reaction was stopped by adding 100 μl of 1.5 N H_2SO_4 to each well. The optical densities at 450 and 650 nm were measured with an ELISA reader (Behring Diagnostic).

Phylogenetic analysis. The putative partial gene sequences of the exo-1,3-ß-glucanases of all *P. insidiosum* isolates (Table 1) were aligned by using the ClustalW program (11), and the alignments were manually edited. The phylogenetic tree of the *P. insidiosum* glucanase gene sequences and the *P. infestans EXO1* sequence (set as the outgroup for rooting) was generated by the neighbor-joining (NJ) algorithm in the Phylip program and drawn using the Treeview program (30). The reliability of the inferred trees was tested using bootstrap resampling with 1,000 replicates (7).

Nucleotide sequence accession numbers. All 22 *P. insidiosum* putative exo-1,3-β-glucanase sequences derived in this study have been submitted to GenBank under accession numbers GU994090 to GU994111 (Table 1).

RESULTS

Mass spectrometric analysis revealed that the 74-kDa antigen is ß-glucanase. Two abundant antigenic proteins, namely, s74 and c74, corresponding to the 74-kDa major immunodominant bands of SABH and CFA, respectively (18), were excised from a one-dimension SDS-polyacrylamide gel to use for PMF by mass spectrometric analysis following trypsin digestion. Comparison of the PMFs of the s74 and c74 antigens did not reveal any significant spectrum similarity (Fig. 1). The peptide masses of each PMF were used to search for theoretical trypsin-digested proteins in the NCBI nonredundant database; and two peptide masses of the s74 antigen, 2,415.3 (relative intensity, 34%) and 2,671.4 (relative intensity, 3%), matched two peptides, WTSIASTQPVGTTTFEHWPIR (s74-1 peptide) and FLTLEEQCDWAFNQYWHLNR (s74-2 peptide), of a putative exo-1,3-ß-glucanase (EXO1) from the plant-pathogenic oomycete *P. infestans* (GenBank accession number AAM18483) (Fig. 1). The s74-1 and s74-2 peptides were matched to amino acid positions 278 to 298 and 634 to 653 of *P. infestans* EXO1, respectively. The *de novo* sequence of the s74-1 peptide determined by MS/MS analysis showed the same amino acid sequence predicted by the initial MS analysis. For the PMFs generated for the c74 protein, no peptide mass matched that of any protein in the NCBI database. Some of the c74 peptide masses with strong relative intensities (i.e., 1,109.6, 1,155.5, 1,238.6, 1,281.6, 1,564.8, and 2,154.1) underwent *de novo* sequencing by MS/MS analysis. The *de novo* amino acid sequences obtained (Table 2) were subjected to BLAST analysis of the sequences in the NCBI database, but none of them matched any protein. Another abundant protein, namely, s43, corresponding to the 43-kDa immunodominant band of SABH (18), was isolated from the SDS-polyacrylamide gel and was

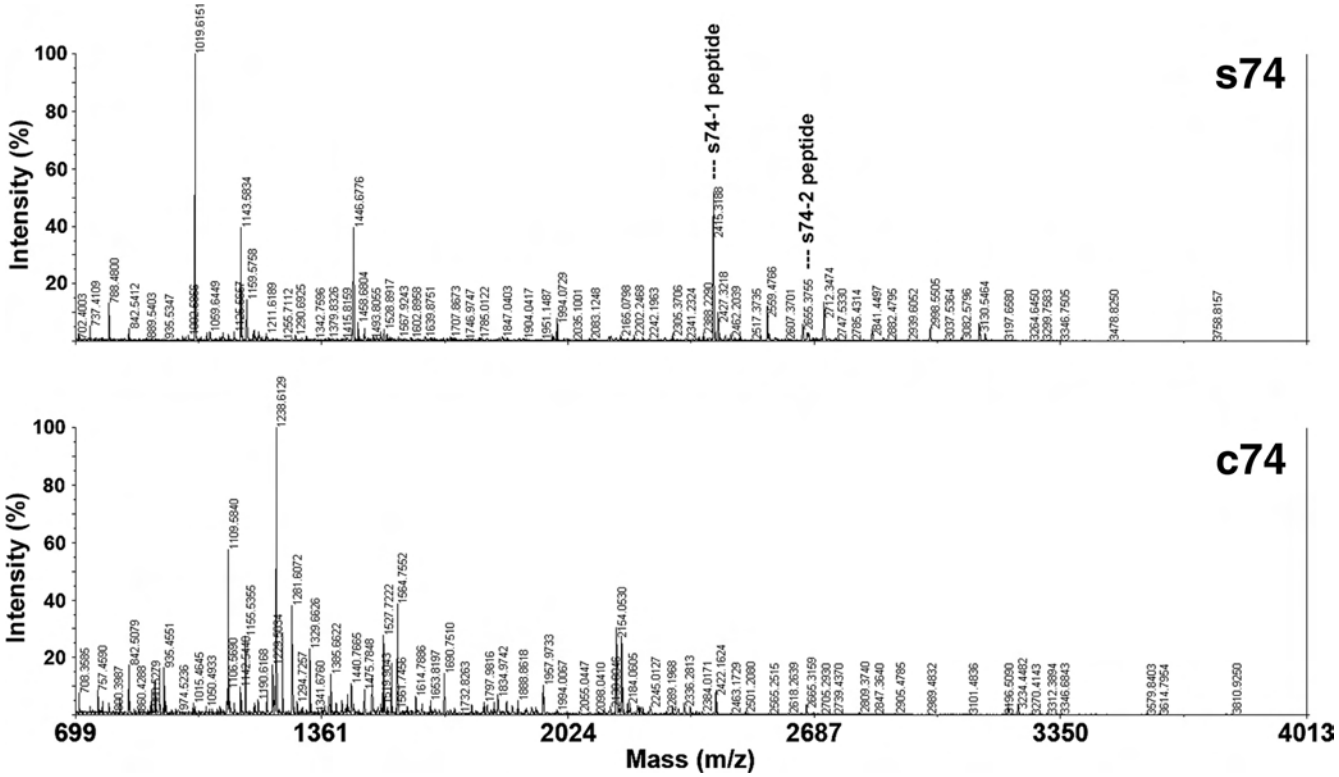

FIG. 1. Peptide mass fingerprints of s74 and c74 immunodominant proteins of *P. insidiosum* after isolation from one-dimension SDS-polyacrylamide gels, in-gel tryptic digestion, and MS analysis. s74-1 and s74-2 were peptides (indicated by the names in the figure) of the s74 protein that matched a putative exo-1,3-ß-glucanase of *Phytophthora infestans* (EXO1).

subjected to the MS and MS/MS analyses. Neither the PMF of this peptide nor the *de novo* sequences of selected peptide masses with strong intensity (i.e., 1,842.8, 1,859.8, and 2,153.0; Table 1) matched any protein in the NCBI database.

Partial DNA sequence of the *P. insidiosum* ß-glucanase gene. PCR primers were designed from within the reverse-translated amino acid sequences of the s74-1 and s74-2 peptides. Degenerate primer Pr1 (128-fold degeneracy) and Pr4 (64-fold degeneracy) were used to amplify the genomic DNA of *P. insidiosum* strains P17, P24, and P40 (Table 1), generating an intense 1.1-kb fragment. The DNA sequences of the 1.1-kb PCR product of each strain were analyzed by BLAST analysis of the sequences in the NCBI nucleotide database (blastn). The highest blastn match (identity, 71%; E value, <1e−146) was to a putative exo-1,3-ß-glucanase gene of *P. infestans* (*EXO1*; GenBank accession number AF494014). The deduced amino acid sequence (363 amino acids) of the partial putative exo-1,3-ß-glucanase gene of *P. insidiosum* amplified from strain P17 had in-frame translations with the s74-1 and s74-2 peptides (Fig. 2A). The amino acid sequence deduced from the PCR product fused with the complete s74-1 and s74-2 peptide sequences (379 amino acids all together) was used for BLAST analysis against the sequences in the NCBI protein database (blastp), and the best match (identity, 73%; positivity, 83%; gap, 0.8%; E value, 8e−170) was an exo-1,3-ß-glucanase protein of *P. infestans* (EXO1; accession number AAM18483) (Fig. 2A). An

TABLE 2. Peptide mass, relative intensity, and BLAST search result for *de novo* amino acid sequences of selected peptide masses of s43, s74, and c74 immunodominant proteins

Protein	Peptide mass	Relative intensity (%)	*De novo* amino acid sequence	BLAST search result
s74	2,415.3	34	WTSIASTQPVGTTTFEHWPIR	Putative exo-1,3-ß-glucanase
c74	1,109.6	55	QMGLNYNPR	No significant similarity found
c74	1,155.5	27	QKAGVSMFDR	No significant similarity found
c74	1,238.6	97	DIAPGMKGMMR	No significant similarity found
c74	1,281.6	36	IYGLADASMKGR	No significant similarity found
c74	1,564.8	37	EGDMNSATGAGMVHR	No significant similarity found
c74	2,154.1	25	KLNVMSGGTTLVTNTAGGG	No significant similarity found
s43	1,842.8	16	HVIMDDISDGTGKR	No significant similarity found
s43	1,859.8	12	CGYVDKGMICGKR	No significant similarity found
s43	2,153.0	>30	MNLQGLGATLATANAHPMPAR	No significant similarity found

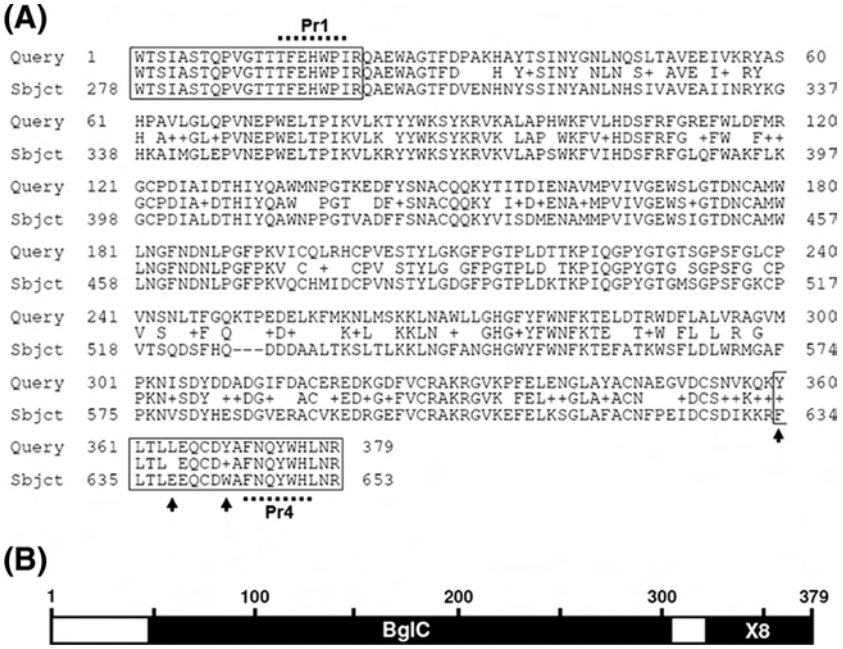

FIG. 2. Protein alignment and conserved domain of the putative glucanase of *P. insidiosum*. (A) Alignment of the deduced amino acid of the s74 partial protein of *P. insidiosm* (query; a 379-amino-acid long protein) and *P. infestans* EXO1 (subject; positions 278 to 653 of the 745-amino-acid long protein). Boxes at the beginning and at the end of the protein sequence indicate the locations of the s74-1 and s74-2 peptides of *P. insidiosum* that were predicted by MS analysis, respectively. Dotted lines at the beginning and at the end of the protein sequence indicate the positions of degenerate primers Pr1 and Pr4, respectively. In respect to the s74-2 peptide sequence of *P. insidiosum*, 3 deduced amino acids at positions 360, 364, and 369 (arrows) were different from the amino acids predicted by MS analysis. (B) Predicted conserved protein domains of the deduced amino acid sequence of the s74 partial protein gene of *P. insidiosum*.

NCBI conserved-domain database search (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) revealed two conserved domains, BglC and the X8 superfamily (Fig. 2B). Similar results were observed for *P. insidiosum* strains P24 and P40.

Immunoreactivity of *P. insidiosum* ß-glucanase peptides. Synthetic peptides s74-1 and s74-2 were each used to coat ELISA plates and tested against the serum samples from patients with pythiosis (samples T1 to T5) and controls (samples C1 to C5). Among the pythiosis sera, pythiosis serum samples T1 (hemagglutination [HA] titer [12] = 1:5,120) and T5 (HA titer = 1:40,960) gave remarkably high ELISA signals (1.2 and 0.8, respectively, for s74-1; 1.2 and 1.0, respectively, for s74-2), while pythiosis serum samples T2 (HA titer = 1:2,560), T3 (HA titer = 1:1,280), and T4 (HA titer = 1:1,280) gave weak to moderate ELISA signals (0.4, 0.3, and 0.3, respectively, for s74-1; 0.5, 0.4, and 0.3, respectively, for s74-2). In general, the s74-1 (Fig. 3A) or the s74-2 (Fig. 3B) peptide gave similar ELISA signal patterns with the different serum samples.

For the s74-1 peptide, the mean ELISA signal of the pythiosis sera was 0.48 (range, 0.26 to 1.17; standard deviation [SD], 0.39), while the mean ELISA signal of the control sera was 0.15 (range, 0.07 to 0.27; SD, 0.09). If a cutoff point of 0.32 (which equals the mean ELISA signal of the control sera plus 2 SDs of the mean) is set, 60% of the pythiosis sera had ELISA signals above this cutoff, but 100% of the control sera had signals below the cutoff (Fig. 3A). For the s74-2 peptide, the mean ELISA signal of the pythiosis sera was 0.57 (range, 0.33 to 1.22; SD, 0.39), while the mean ELISA signal of the control sera was 0.09 (range, 0.05 to 0.18; SD, 0.06). On the basis of the cutoff point of 0.21 (the mean ELISA signal of control sera plus 2 SDs), all pythiosis sera were clearly differentiated from all control sera (Fig. 3B).

Phylogenetic analysis of *P. insidiosum* ß-glucanase genetic sequences. The ß-glucanase gene sequences of 22 *P. insidiosum* isolates from patients with different forms of pythiosis who lived in different regions of Thailand (Table 1) were successfully amplified and sequenced using primers Pr1 and Pr4 or primers Pr7 and Pr10. These sequences were analyzed for phylogenetic relatedness using the NJ algorithm (setting the *P. infestans EXO1* gene as an outgroup for rooting). From this analysis, *P. insidiosum* can be divided into two phylogenetic groups, clade A and clade B, with 98% and 100% reliability, respectively (Fig. 4). The distributions of the forms of pythiosis and the geographic origins of the patients in the two clades were similar.

DISCUSSION

Several proteins with immunodominant reactivity against pythiosis patient sera were analyzed by mass spectrometry of tryptic digests to determine their protein identities. These analyses included proteins corresponding to the 74- and 43-kDa antigens of *P. insidiosum* cytoplasmic proteins (SABH; s74 and s43 antigens) and the 74-kDa antigen of secreted proteins (CFA; c74 antigen) (18). The topologies of the PMFs of the s74 and c74 antigens were different (Fig. 1), suggesting that although these antigens coincidentally had the same molecular size (74 kDa) and are major immunodominant anti-

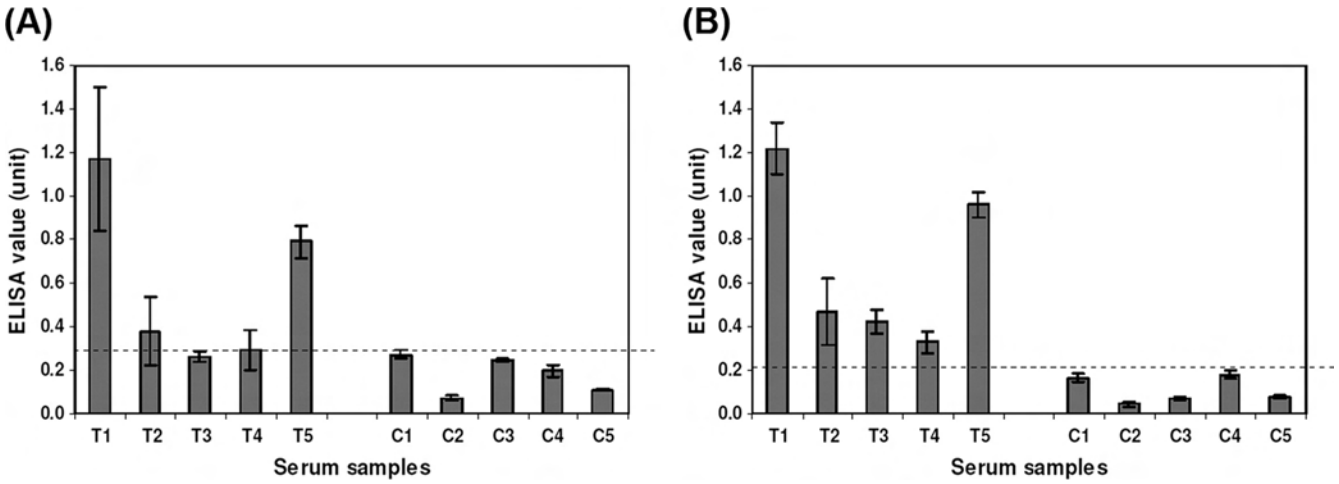

FIG. 3. Immunoreactivity of s74-1 (A) and s74-2 (B) peptides against pythiosis sera (samples T1 to T5) and control sera (samples C1 to C5) by ELISA. For the s74-1 peptide, the ELISA cutoff point (dashed line; the mean ELISA signal of the control sera plus 2 SDs) is 0.32, and the mean ELISA signals of the pythiosis and control sera are 0.48 (range, 0.26 to 1.17; SD, 0.39) and 0.15 (range, 0.07 to 0.27; SD, 0.09), respectively. For the s74-2 peptide, the ELISA cutoff point is 0.21 and the mean ELISA signal of the pythiosis and control sera are 0.57 (range, 0.33 to 1.22; SD, 0.39) and 0.09 (range, 0.05 to 0.18; SD, 0.06), respectively.

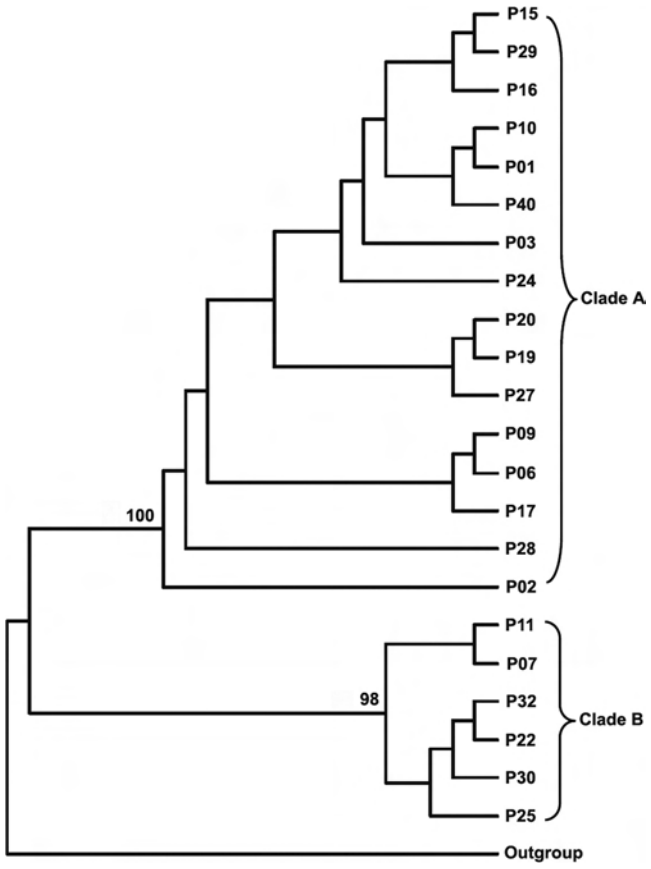

FIG. 4. Phylogenetic analysis of 22 clinical isolates of *P. insidiosum*, based on partial putative exo-1,3-ß-glucanase DNA sequences, reveals that the organism can be divided into two clades, clade A ($n = 16$) and clade B ($n = 6$). *P. infestans EXO1* is used as an outgroup for rooting.

gens, they were not the same protein. For the peptide masses of the s74 antigen, two matches were found by searching for theoretical trypsin-digested proteins in the NCBI nonredundant protein database. These two peptide masses of the s74 antigen, s74-1 and s74-2, had predicted amino acid compositions identical to those of the predicted tryptic peptides of EXO1 of *P. infestans*, an oomycete member in a genus closely related to *Pythium*. The s74-1 peptide was selected for *de novo* sequencing because from the s74 PMF, it had a stronger intensity than the s74-2 peptide (34% and 3%, respectively). The *de novo* sequence of the 74-1 peptide amino acid sequence perfectly matched that of *P. infestans* EXO1, confirming that the predicted amino acid sequence of this peptide determined by the MS analysis was correct, and thus, the identity of the s74 protein, a putative glucanase, was confirmed. Some of the strong-intensity peptide masses of c74 and s43 proteins (Table 2) were selected for *de novo* sequencing. None of these additional *de novo* sequences matched the sequence of any protein in the protein database; therefore, these peptides may be unique to *P. insidiosum*.

For *P. infestans* EXO1 (745 amino acids long), the sequence encompassing the s74-1 and s74-2 peptides and the region in between is predicted to be 376 amino acids, and the length of corresponding DNA sequence is predicted to be 1,128 bp long. This prediction is consistent with the fact that the degenerate primers (Pr1 and Pr4) designed from the s74-1 and s74-2 peptide sequences successfully amplified a 1.1-kb PCR product, and the DNA sequence significantly matched that of *P. infestans EXO1* (data not shown). The amino acid sequence deduced from this *P. insidiosum* DNA sequence had high degrees of identity and similarity to the *P. infestans* EXO1 peptide over its entire length (Fig. 2A). Searches of NCBI conserved-domain databases revealed that the putative *P. insidisoum* glucanase had two predicted conserved domains, namely, BglC and X8 (Fig. 2B). These domains are found in proteins involved in carbohydrate transportation, metabolism, and bind-

ing. Three consecutive amino acids corresponding to amino acid positions 250 to 252 of the *P. insidiosum* glucanase were absent in the *P. infestans* EXO1, making this portion of *P. infestans* EXO1 a little shorter (Fig. 2A). In respect to the s74-2 peptide sequence derived from the MS analysis, there were 3 discrepant amino acids found in the *P. insidiosum* deduced amino acid sequences, at positions 360, 364, and 369 (indicated by arrows in Fig. 2A). In spite of this error in the sequence prediction by the MS analysis, the proteomic, molecular genetic, and bioinformatic evidence consistently supports the conclusion that the 74-kDa protein of *P. insidiosum* is an exo-1,3-ß-glucanase. Glucanases are an interesting group of hydrolytic enzymes that are present in many organisms, including fungi (2, 22, 33). Glucans are major cell wall components that give strength and rigidity to fungal cells, and fungal glucanase hydrolytic activity is necessary in order to remodel the cell wall for hyphal elongation or growth (2, 22, 33). Although putative glucanases have been identified in some oomycetes (8, 23), little is know about their biological and pathological roles. In the pathogenic fungus *Candida albicans* (21, 35), glucanases have roles in morphogenesis (hyphal formation and growth), pathogenesis (adhesion and hyphal invasion), and host immune responses (stimulation of host immunity).

Although the characterization of the glucanase of *P. insidiosum* began with a 74-kDa major immunodominant band in the Western blot analysis (18), we sought further evidence for its immunoreactivity. To provide a specific test for immune recognition, an ELISA was developed using either the s74-1 or s74-2 synthetic peptide of the glucanase. The mean ELISA signal for the pythiosis sera was higher than that of the control sera for both the s74-1 and the s74-2 peptides (3.2-fold and 6.3-fold higher, respectively). The ELISA results for individual pythiosis sera were higher than those for all of the control sera in 60% of the cases using the s74-1 peptide and 100% of the cases using the s74-2 peptide (Fig. 3). These results confirm the immunoreactivity of the *P. insidiosum* glucanase and indicate that the s74-2 peptide is more robustly immunoreactive than the s74-1 peptide in ELISAs. Furthermore, the high ELISA signals for serum samples T1 and T5 correlated with their high HA titers, while the low ELISA signals for serum samples T2, T3, and T4 correlated with the low HA titers of these sera (Fig. 3). Further optimizations of the ELISA by increasing the purity and the concentration of each peptide might improve the assay discrimination power, a factor necessary for development of a serodiagnostic test.

Use of the exo-1,3-ß-glucanase gene sequence of *P. insidiosum* for phylogenetic analysis revealed two separable groups (clades A and B) of *P. insidiosum* in Thailand (Fig. 4). This finding is consistent with previous rDNA sequence analyses that also showed two clades for *P. insidiosum* in Thailand (5, 40, 41). It is noteworthy that for both glucanase- and rDNA-based analyses, there is no correlation of the phylogenetic group with either the geographic distribution or the clinical presentation of pythiosis patients.

Because other oomycetes have ß-glucan as a major cell wall component (20), it is expected that *P. insidiosum* would as well. The discovery, in *P. insidiosum*, of a putative glucanase (a glucan-hydrolyzing enzyme, which may be necessary for cell wall remodeling in the fungi [33, 35]) further supports this possibility. The presence of ß-glucan in their cell wall could mean that the oomycetes would be sensitive to a new class of antifungal drugs, the echinocandins (i.e., caspofungin, anidulafungin, and micafungin), whose mechanism of antifungal action is to inhibit cell wall ß-glucan synthesis (1). However, in both *in vivo* and *in vitro* experiments, caspofungin failed to adequately control the growth of *P. insidiosum* (3, 32). The reason for this failure is unknown but could result from differences between the fungal and oomycete glucan synthases or from other differences, such as the uptake, binding, or metabolism of the drug.

In the present study, we have successfully used a proteomic approach to characterize a 74-kDa major immunoreactive antigen of *P. insidiosum* as a putative exo-1,3-ß-glucanase. Molecular genetic analysis showed that this protein was encoded by a gene sequence homologous to a *P. infestans* exo-1,3-ß-glucanase gene, *EXO1*. The *P. insidiosum EXO1* DNA sequence can be classified into two phylogenetic groups. Because the s74-1 and s74-2 peptides, representing portions of the *P. insidiosum* 74-kDa glucanase, were strongly recognized by sera from patients with pythiosis (Fig. 3), they could be new antigenic targets in the development of serodiagnostic tests. The *P. insidiosum* 74-kDa glucanase could also be tested as a new vaccine candidate in an established animal model of pythiosis (36).

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REFERENCES

1. **Abuhammour, W., and E. Habte-Gaber.** 2004. Newer antifungal agents. Indian J. Pediatr. **71:**253–259.
2. **Adams, D. J.** 2004. Fungal cell wall chitinases and glucanases. Microbiology **150:**2029–2035.
3. **Brown, T. A., A. M. Grooters, and G. L. Hosgood.** 2008. In vitro susceptibility of *Pythium insidiosum* and a *Lagenidium* sp to itraconazole, posaconazole, voriconazole, terbinafine, caspofungin, and mefenoxam. Am. J. Vet. Res. **69:**1463–1468.
4. **Brown, C. C., J. J. McClure, P. Triche, and C. Crowder.** 1988. Use of immunohistochemical methods for diagnosis of equine pythiosis. Am. J. Vet. Res. **49:**1866–1868.
5. **Chaiprasert, A., T. Krajaejun, S. Pannanusorn, C. Prariyachatigul, W. Wanachiwanawin, B. Sathapatayavongs, T. Juthayothin, N. Smittipat, N. Vanittanakom, and A. Chindamporn.** 2009. *Pythium insidiosum* Thai isolates: molecular phylogenetic analysis. Asian Biomed. **3:**623–633.
6. **Chaiprasert, A., S. Samerpitak, W. Wanachiwanawin, and P. Thasnakorn.** 1990. Induction of zoospore formation in Thai isolates of *Pythium insidiosum*. Mycoses **33:**317–323.
7. **Felsenstein, J.** 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution **39:**783–791.
8. **Gaulin, E., M. A. Madoui, A. Bottin, C. Jacquet, C. Mathé, A. Couloux, P. Wincker, and B. Dumas.** 2008. Transcriptome of Aphanomyces euteiches: new oomycete putative pathogenicity factors and metabolic pathways. PLoS One **3:**e1723.
9. **Grooters, A. M., and M. K. Gee.** 2002. Development of a nested polymerase chain reaction assay for the detection and identification of *Pythium insidiosum*. J. Vet. Intern. Med. **16:**147–152.
10. **Grooters, A. M., B. S. Leise, M. K. Lopez, M. K. Gee, and K. L. O'Reilly.** 2002. Development and evaluation of an enzyme-linked immunosorbent assay for the serodiagnosis of pythiosis in dogs. J. Vet. Intern. Med. **16:**142–146.
11. **Higgins, D., J. Thompson, and T. Gibson.** 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence

weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. **22:**4673–4680.
12. **Jindayok, T., S. Piromsontikorn, S. Srimuang, K. Khupulsup, and T. Krajaejun.** 2009. Hemagglutination test for rapid serodiagnosis of human pythiosis. Clin. Vaccine Immunol. **16:**1047–1051.
13. **Kamoun, S.** 2003. Molecular genetics of pathogenic oomycetes. Eukaryot. Cell **2:**191–199.
14. **Keeling, P. J., G. Burger, D. G. Durnford, B. F. Lang, R. W. Lee, R. E. Pearlman, A. J. Roger, and M. W. Gray.** 2005. The tree of eukaryotes. Trends Ecol. Evol. **20:**670–676.
15. **Keeratijarut, A., P. Karnsombut, R. Aroonroch, S. Srimuang, T. Sangruchi, L. Sansopha, P. Mootsikapun, N. Larbcharoensub, and T. Krajaejun.** 2009. Evaluation of an in-house immunoperoxidase staining assay for histodiagnosis of human pythiosis. Southeast Asian J. Trop. Med. Public Health **40:**1298–1305.
16. **Krajaejun, T., S. Imkhieo, A. Intaramat, and K. Ratanabanangkoon.** 2009. Development of an immunochromatographic test for rapid serodiagnosis of human pythiosis. Clin. Vaccine Immunol. **16:**506–509.
17. **Krajaejun, T., M. Kunakorn, S. Niemhom, P. Chongtrakool, and R. Pracharktam.** 2002. Development and evaluation of an in-house enzyme-linked immunosorbent assay for early diagnosis and monitoring of human pythiosis. Clin. Diagn. Lab. Immunol. **9:**378–382.
18. **Krajaejun, T., M. Kunakorn, R. Pracharktam, P. Chongtrakool, B. Sathapatayavongs, A. Chaiprasert, N. Vanittanakom, A. Chindamporn, and P. Mootsikapun.** 2006. Identification of a novel 74-kilodalton immunodominant antigen of *Pythium insidiosum* recognized by sera from human patients with pythiosis. J. Clin. Microbiol. **44:**1674–1680.
19. **Krajaejun, T., B. Sathapatayavongs, R. Pracharktam, P. Nitiyanant, P. Leelachaikul, W. Wanachiwanawin, A. Chaiprasert, P. Assanasen, M. Saipetch, P. Mootsikapun, P. Chetchotisakd, A. Lekhakula, W. Mitarnun, S. Kalnauwakul, K. Supparatpinyo, R. Chaiwarith, S. Chiewchanvit, N. Tananuvat, S. Srisiri, C. Suankratay, W. Kulwichit, M. Wongsaisuwan, and S. Somkaew.** 2006. Clinical and epidemiological analyses of human pythiosis in Thailand. Clin. Infect. Dis. **43:**569–576.
20. **Kwon-Chung, K. J.** 1994. Phylogenetic spectrum of fungi that are pathogenic to humans. Clin. Infect. Dis. **19:**S1–S7.
21. **La Valle, R., S. Sandini, M. J. Gomez, F. Mondello, G. Romagnoli, R. Nisini, and A. Cassone.** 2000. Generation of a recombinant 65-kilodalton mannoprotein, a major antigen target of cell-mediated immune response to Candida albicans. Infect. Immun. **68:**6777–6784.
22. **Martin, K., B. M. McDougall, S. McIlroy, J. Chen, and R. J. Seviour.** 2007. Biochemistry and molecular biology of exocellular fungal beta-(1,3)- and beta-(1,6)-glucanases. FEMS Microbiol. Rev. **31:**168–192.
23. **McLeod, A., C. D. Smart, and W. E. Fry.** 2003. Characterization of 1,3-beta-glucanase and 1,3;1,4-beta-glucanase genes from Phytophthora infestans. Fungal Genet. Biol. **38:**250–263.
24. **Mendoza, L., L. Ajello, and M. R. McGinnis.** 1996. Infection caused by the oomycetous pathogen *Pythium insidiosum*. J. Mycol. Med. **6:**151–164.
25. **Mendoza, L., F. Hernandez, and L. Ajello.** 1993. Life cycle of the human and animal oomycete pathogen *Pythium insidiosum*. J. Clin. Microbiol. **31:**2967–2973.
26. **Mendoza, L., L. Kaufman, W. Mandy, and R. Glass.** 1997. Serodiagnosis of human and animal pythiosis using an enzyme-linked immunosorbent assay. Clin. Diagn. Lab. Immunol. **4:**715–718.
27. **Mendoza, L., L. Kaufman, and P. G. Standard.** 1986. Immunodiffusion test for diagnosing and monitoring pythiosis in horses. J. Clin. Microbiol. **23:**813–816.
28. **Mendoza, L., and J. C. Newton.** 2005. Immunology and immunotherapy of the infections caused by *Pythium insidiosum*. Med. Mycol. **43:**477–486.
29. **Mendoza, L., and J. Prendas.** 1988. A method to obtain rapid zoosporogenesis of *Pythium insidiosum*. Mycopathologia **104:**59–62.
30. **Page, R. D. M.** 1996. TREEVIEW: an application to display phylogenetic trees on personal computers. Comput. Appl. Biosci. **12:**357–358.
31. **Pannanusorn, S., A. Chaiprasert, C. Prariyachatigul, T. Krajaejun, N. Vanittanakom, A. Chindamporn, W. Wanachiwanawin, and B. Satapatayavong.** 2007. Random amplified polymorphic DNA typing and phylogeny of *Pythium insidiosum* clinical isolates in Thailand. Southeast Asian J. Trop. Med. Public Health **38:**383–391.
32. **Pereira, D. I., J. M. Santurio, S. H. Alves, J. S. Argenta, L. Pötter, A. Spanamberg, and L. Ferreiro.** 2007. Caspofungin in vitro and in vivo activity against Brazilian *Pythium insidiosum* strains isolated from animals. J. Antimicrob. Chemother. **60:**1168–1171.
33. **Pitson, S. M., R. J. Seviour, and B. M. McDougall.** 1993. Noncellulolytic fungal beta-glucanases: their physiology and regulation. Enzyme Microb. Technol. **15:**178–192.
34. **Pracharktam, R., P. Changtrakool, B. Sathapatayavongs, P. Jayanetra, and L. Ajello.** 1991. Immunodiffusion test for diagnosis and monitoring of human pythiosis insidiosi. J. Clin. Microbiol. **29:**2661–2662.
35. **Sandini, S., R. La Valle, F. De Bernardis, C. Macrì, and A. Cassone.** 2007. The 65 kDa mannoprotein gene of Candida albicans encodes a putative beta-glucanase adhesion required for hyphal morphogenesis and experimental pathogenicity. Cell. Microbiol. **9:**1223–1238.
36. **Santurio, J. M., A. T. Leal, A. B. Leal, R. Festugatto, I. Lubeck, E. S. Sallis, M. V. Copetti, S. H. Alves, and L. Ferreiro.** 2003. Three types of immunotherapics against pythiosis insidiosi developed and evaluated. Vaccine **21:**2535–2540.
37. **Saveliev, S., D. Simpson, W. Daily, C. Woodroofe, D. Klaubert, G. Sabat, R. Bulliet, and K. V. Wood.** 2008. Improve protein analysis with the new, mass spectrometry-compatible ProteasMAX™ surfactant. Promega Notes **99:**3–7.
38. **Schlosser, E., and D. Gottlieb.** 1966. Sterols and the sensitivity of *Pythium* species to filipin. J. Bacteriol. **91:**1080–1084.
39. **Schurko, A. M., L. Mendoza, A. W. de Cock, J. E. Bedard, and G. R. Klassen.** 2004. Development of a species-specific probe for *Pythium insidiosum* and the diagnosis of pythiosis, J. Clin. Microbiol. **42:**2411–2418.
40. **Schurko, A. M., L. Mendoza, C. A. Lévesque, N. L. Désaulniers, A. W. de Cock, and G. R. Klassen.** 2003. A molecular phylogeny of *Pythium insidiosum*. Mycol. Res. **107:**537–544.
41. **Supabandhu, J., M. C. Fisher, L. Mendoza, and N. Vanittanakom.** 2008. Isolation and identification of the human pathogen *Pythium insidiosum* from environmental samples collected in Thai agricultural areas. Med. Mycol. **46:**41–52.
42. **Supabandhu, J., P. Vanittanakom, K. Laohapensang, and N. Vanittanakom.** 2009. Application of immunoblot assay for rapid diagnosis of human pythiosis. J. Med. Assoc. Thai. **92:**1063–1071.
43. **Vanittanakom, N., J. Supabandhu, C. Khamwan, J. Praparattanapan, S. Thirach, N. Prasertwitayakij, W. Louthrenoo, S. Chiewchanvit, and N. Tananuvat.** 2004. Identification of emerging human-pathogenic *Pythium insidiosum* by serological and molecular assay-based methods. J. Clin. Microbiol. **42:**3970–3974.
44. **Wanachiwanawin, W., L. Mendoza, S. Visuthisakchai, P. Mutsikapan, B. Sathapatayavongs, A. Chaiprasert, P. Suwanagool, W. Manuskiatti, C. Ruangsetakit, and L. Ajello.** 2004. Efficacy of immunotherapy using antigens of *Pythium insidiosum* in the treatment of vascular pythiosis in humans. Vaccine **22:**3613–3621.

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Development of an Immunochromatographic Test for Rapid Serodiagnosis of Human Pythiosis▽

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Human pythiosis is an emerging and life-threatening infectious disease caused by the fungus-like organism *Pythium insidiosum*. High rates of morbidity and mortality for patients with pythiosis are exacerbated by the lack of early diagnosis and an effective treatment. Here, we developed and evaluated an immunochromatographic test (ICT) for the diagnosis of human pythiosis, in comparison to a standard serological test of immunodiffusion (ID). Culture filtrate antigen of *P. insidiosum* was used to detect human anti-*P. insidiosum* antibody. Sheep anti-human immunoglobulin G-colloidal gold conjugate was used to generate an ICT signal. Thirty-three sera from patients with vascular ($n = 27$), ocular ($n = 4$), and cutaneous ($n = 2$) pythiosis and 181 control sera from healthy blood donors ($n = 100$), as well as patients with a variety of infectious ($n = 56$) and noninfectious ($n = 25$) diseases, were included in the test evaluation. The turnaround time for generating a result by the ICT was less than 30 min, while that for ID was ~24 h. Based on the results for all sera of pythiosis patients and the control groups, the ICT showed 88% sensitivity and 100% specificity and ID showed 61% sensitivity and 100% specificity. By both tests, false-negative results for sera from all ocular pythiosis patients were obtained. In addition, the ID test yielded false-negative results for sera from eight patients with vascular pythiosis and one patient with cutaneous pythiosis. It was concluded that the ICT is a rapid, user-friendly, and reliable serological test for the early diagnosis of vascular and cutaneous pythiosis.

Pythiosis is a life-threatening infectious disease caused by the oomycete, fungus-like, aquatic organism *Pythium insidiosum*, which is the only *Pythium* species of the kingdom *Stramenopila* known to infect humans and some animals, such as horses, dogs, cats, and cattle, in tropical and subtropical countries (5, 11). Although microscopic features of oomycete organisms are similar to those of fungi, a phylogenic analysis shows that *Pythium* spp. are more closely related to diatoms and algae than to the true fungi (10). *P. insidiosum* inhabits swampy areas, where it exists in two stages: perpendicular branching hyphae and biflagellate zoospores (12). Infection has been proposed to occur by invasion of the zoospores into host tissue after attachment and germination (12).

Human pythiosis is endemic in Thailand, where the disease has been increasingly reported from all over the country (2, 3, 8, 9, 19–24, 26, 27). Four forms of human pythiosis have been described: (i) cutaneous pythiosis, affecting the face or limbs as a granulomatous and ulcerating lesion; (ii) vascular pythiosis, affecting arteries and resulting in arterial occlusion or an aneurysm; (iii) ocular pythiosis, causing corneal ulcers; and (iv) disseminated pythiosis, featuring the infection of internal organ (9). Vascular and ocular infections are the most common forms of pythiosis. The majority of vascular pythiosis patients have an affected leg amputated, while most ocular pythiosis patients have an infected eye removed (9). Many vascular pythiosis patients die from a ruptured aneurysm. Thalassemias and agriculture-related careers are known as predisposing factors (9, 21, 27).

Culture identification is a definite diagnostic method for pythiosis, but it is a time-consuming procedure and requires expertise and often hard-to-obtain internal tissue (1, 9, 11, 17, 23). Conventional antifungal drugs are not effective to control the infection (9). The main treatment option for pythiosis is surgery, which should be urgently performed to limit disease progression and ensure better prognoses for patients (9). Some serodiagnostic tests have been developed to facilitate the early diagnosis of pythiosis (4, 6, 7, 13–15, 18, 25). In-house enzyme-linked immunosorbent and Western blot assays show high degrees of sensitivity and specificity for the diagnosis of pythiosis (6, 7, 13). However, the tests require skilled personnel, stable and reproducible reagents, expensive equipment, and long turnaround times. Immunodiffusion (ID) (4, 14, 18) is a simple serological test that has been commonly used in laboratories for the diagnosis of pythiosis and is considered to be a standard serodiagnostic test for pythiosis. Although the ID test is easy to perform and has high specificity, it shows poor sensitivity and requires a long turnaround time, which may lead to a false-negative result and delayed treatment. Therefore, improvement in the diagnostic procedure is an important health care goal.

The immunochromatographic test (ICT) has been popularly applied for the serodiagnosis of many infectious diseases because of its user-friendly format, rapid result generation, and high degrees of detection sensitivity and specificity. Most im-

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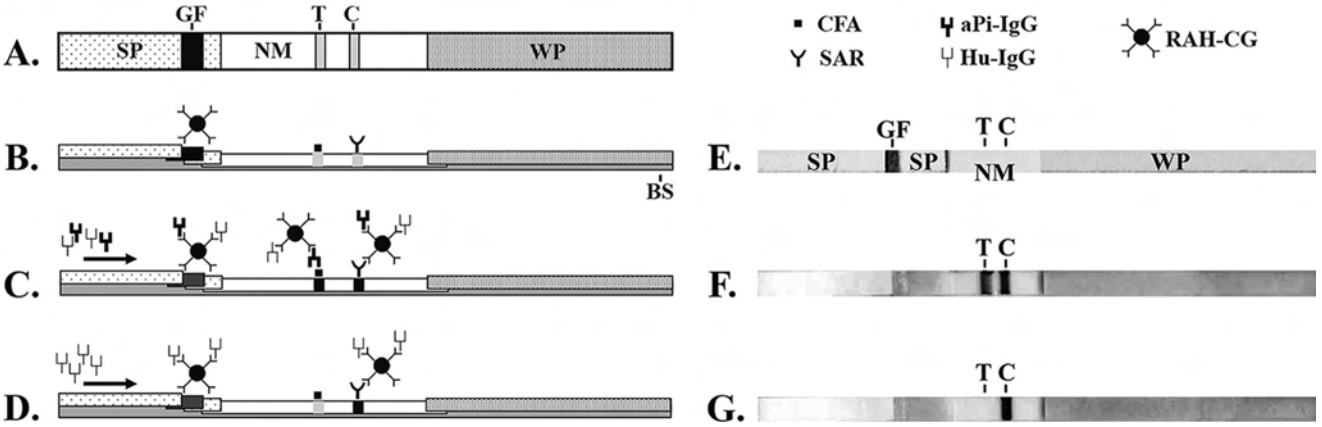

FIG. 1. Schematic diagrams of an ICT strip: top view (A, E, F, and G) and side view (B, C, and D). (A and B) An ICT strip consists of a plastic backing support (BS), two sample pads (SP), a glass fiber (GF; containing rabbit anti-human IgG-colloidal gold conjugate), a nitrocellulose membrane (NM; containing test [T] and control [C] lines), and a wicking pad (WP). (C) Positive result. The test and control lines are visible. (D) Negative result. Only the control line is visible. (E) Actual ICT strip corresponding to the diagrams in panels A and B. (F) Actual ICT strip with a positive result, corresponding to the diagram in panel C. (G) Actual ICT strip with a negative result, corresponding to the diagram in panel D. Arrows show the direction of serum flow. SAR, sheep anti-rabbit antibody; aPi-IgG, anti-*P. insidiosum* IgG; Hu-IgG, human IgG; RAH-CG, rabbit anti-human IgG-colloidal gold conjugate.

portantly, the test can be used in remote areas or areas where pythiosis is endemic which lack diagnostic facilities. In the present study, we aimed to develop an in-house ICT for the rapid detection of specific human anti-*P. insidiosum* immunoglobulin G (IgG) in serum samples. The performance of the ICT was evaluated in comparison to that of an ID test for the serodiagnosis of pythiosis.

MATERIALS AND METHODS

Microorganism and growth conditions. The *P. insidiosum* strain CBS119452, isolated from Thai patients with vascular pythiosis, was used to prepare antigen in this study. The organism had been maintained on Sabouraud dextrose agar at 37°C until antigen preparation.

Antigen preparation. The *P. insidiosum* CBS119452 isolate was subcultured on Sabouraud dextrose agar and incubated at 37°C for 2 days. Several small agar pieces containing hyphal elements from the growing culture were transferred into 200 ml of Sabouraud dextrose broth and shaken (150 rpm) at 37°C for 1 week. Thimerosal (Merthiolate; final concentration, 0.02% [wt/vol]) was added to kill the cultures before they were filtered through a Durapore membrane filter (0.22-μm pore size; Millipore, County Cork, Ireland). 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 Ultra-15 centrifugal filter (nominal molecular weight limit, 10,000; Millipore, Bedford, MA). The concentrated filtered broth was referred to as culture filtrate antigen (CFA) and was measured for protein concentration by a spectrophotometer. The CFA was stored at −20°C until use.

Serum samples. A total of 33 sera from patients with known cases of human pythiosis (27 vascular, 4 ocular, and 2 cutaneous) were used for the test evaluation. The diagnosis of human pythiosis was based on previously reported criteria (9), as follows: (i) culture isolation of *P. insidiosum* ($n = 15$), (ii) serodiagnosis ($n = 9$), and (iii) the presence of the unique clinicopathological features of vascular pythiosis ($n = 9$). An additional 181 sera were collected for use in four control groups. The first group included 100 sera randomly collected from healthy blood donors who came to the Blood Bank Division, Ramathibodi Hospital. The second group included sera from 19 healthy thalassemic patients with no clinical evidence of pythiosis. The third group included sera from six patients with noninfectious diseases (five with highly positive antinuclear antibody titers and one with thromboangiitis obliterans [TAO]). The fourth group included sera from 56 patients with other infections (7 with penicilliosis, 7 galactomannan positive, 6 with cryptococcosis, 5 with malaria, 4 with aspergillosis, 4 with mycoplasmosis, 3 with zygomycosis, 3 with histoplasmosis, 3 with syphilis, 3 positive for anti-human immunodeficiency virus [HIV] antibody, 2 with toxoplasmosis, 2 with leptospirosis, 2 with melioidosis, 1 with amoebiasis, 1 with disseminated candidiasis, 1 positive for anti-hepatitis A virus antibody, 1 positive for anti-hepatitis B virus antibody, and 1 positive for anti-hepatitis C virus antibody). All sera were kept at −20°C until use.

ICT. (i) Conjugation of antibody to colloidal gold. The 40-nm-particle colloidal gold suspension (Arista, Allentown, PA) was adjusted to pH 9.65 by using 0.2 M Na_2CO_3. To each 500 μl of colloidal gold, 3 μg of rabbit anti-human IgG (Dako, Glostrup, Denmark) was added, and the mixture was incubated for 30 min at room temperature. The residual surfaces of the colloidal gold particles were blocked by incubation with 5% (wt/vol) bovine serum albumin (Sigma, St. Louis, MO) for 10 min. The conjugate was centrifuged at 6,000 × *g* for 15 min, and the supernatant was then discarded. The conjugate pellet was washed in 0.5% (wt/vol) casein and centrifuged at 6,000 × *g* for 15 min. After the removal of the supernatant, the conjugate was resuspended in a solution of 0.5% (wt/vol) casein and 20% (wt/vol) sucrose in 0.02 M Tris-HCl (pH 8.0) with 40 times less volume than the original suspension. A piece of 2.5- by 2.5-mm glass fiber (GF33; Whatman Schleicher & Schuell, Dassel, Germany) was impregnated with this IgG-colloidal gold conjugate (2.5 μl) and dried in a dehumidifier cabinet for an hour.

(ii) Immobilization of antigen and antibody onto a nitrocellulose membrane. A 1.5-cm-wide nitrocellulose membrane (AE99; Whatman Schleicher & Schuell, Dassel, Germany) was lined with CFA (1:5 dilution; the test line) and sheep anti-rabbit IgG (150 μg/ml in 50 mM ammonium acetate buffer, pH 4.5; the control line) at 1 μl/cm by a dispenser (ZX 1000; BioDot, Irvine, CA) (Fig. 1A and B). The membrane was dried, blocked with 1% (wt/vol) bovine serum albumin, and dried again in a dehumidifier cabinet.

(iii) Assembly of ICT strips. The immobilized nitrocellulose membrane, the glass fiber with the colloidal gold conjugate, the sample pad (903 paper; Whatman Schleicher & Schuell, Dassel, Germany), and the wicking pad (3MM chromatography paper; Whatman, Maidstone, England) were assembled onto a backing of plastic, which was then cut into 2.5-mm-wide strips by a strip-cutting machine (CM 4000 R; BioDot, Irvine, CA) (Fig. 1A and B).

(iv) Detection of human anti-*P. insidiosum* antibody by the ICT. Each individual sample was diluted 1:10,000 in phosphate buffer (pH 7.4). The ICT was evaluated in duplicate with 100 μl of diluted serum in a 96-well microtiter plate. The test signal of each ICT strip was read by the naked eye at 30 min by three independent laboratory personnel. To quantify the ICT signal, each strip was scanned by a scanner (Epson Perfection 1670 photo scanner; Seiko Epson Corp., Japan) to obtain a tagged image file format picture. Test and background signal intensities were measured by the Quantity-One program (Bio-Rad). The intensity value derived from a test signal after the subtraction of the background signal was referred to as the ICT value (IV). Sensitivities and specificities for all cutoff levels of IVs were calculated and graphically displayed in receiver-operating characteristic (ROC) curves by using the Stata version 10 program (StataCorp, TX).

ID test. The ID test was modified from the method of Pracharktam et al. (18). Briefly, agar gel diffusion was carried out on a 5-cm-diameter petri dish with 2%

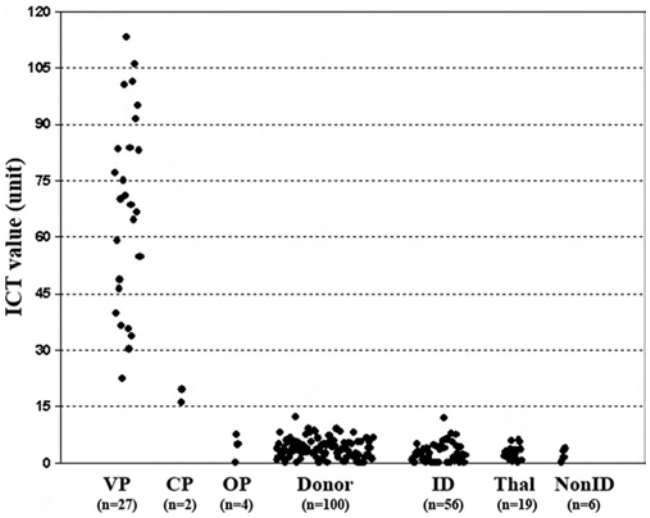

FIG. 2. IVs of all sera from patients with vascular pythiosis (VP), cutaneous pythiosis (CP), and ocular pythiosis (OP), from blood donors, and from patients with a variety of infectious diseases (ID) and noninfectious diseases, including thalassemia (Thal) and other noninfectious diseases (nonID).

agar in barbital (Veronal) buffer (0.9% [wt/vol] $C_8H_{11}N_2NaO_3$, 0.05% [wt/vol] NaN_3, pH 8.6). The CFA and serum to be tested were each added to 4-mm-diameter wells separated by 4 mm. The petri dish was incubated in a moist chamber at room temperature for 24 h. The appearance of a precipitation line visible to the naked eye was considered a positive test result.

RESULTS

Development of the ICT results. The components of an ICT strip are depicted in Fig. 1. CFA was blotted onto a nitrocellulose membrane (indicated as the test line) and used as the specific *P. insidiosum* antigen for detecting anti-*P. insidiosum* IgG in serum samples. Sheep anti-rabbit IgG (indicated as the control line) was blotted distally from CFA. When human IgGs in serum moved upward by capillary action through the glass fiber, they formed complexes with the rabbit anti-human IgG-colloidal gold conjugate. The complexes migrated through the nitrocellulose membrane. Immune complexes containing human anti-*P. insidiosum* IgG bound CFA and developed a purple signal at the test line. In contrast, immune complexes lacking human anti-*P. insidiosum* IgG passed through the test line without developing a signal. The sheep anti-rabbit IgG bound the remaining immune complexes containing rabbit anti-human IgG-colloidal gold conjugate and exhibited an internal test validation signal at the control line.

Diagnostic performance of the ICT in comparison to ID. ICT and ID results were read by three independent laboratory personnel. Based on the results for all sera from pythiosis patients (27 with vascular pythiosis, 4 with ocular pythiosis, and 2 with cutaneous pythiosis) and the control groups, the ICT showed 88% sensitivity, 100% specificity, 100% positive predictive value, and 98% negative predictive value while ID showed 61% sensitivity, 100% specificity, 100% positive predictive value, and 93% negative predictive value. False-negative ICT results were obtained for sera from all ocular pythiosis patients. False-negative ID results were obtained for sera from all ocular pythiosis patients, eight patients with vascular pythiosis, and one patient with cutaneous pythiosis.

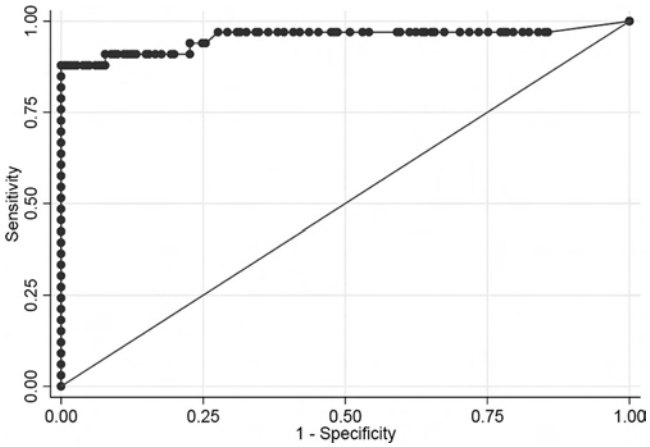

FIG. 3. ROC curve. Pythiosis and control serum groups (total, 214 samples) are included in the ROC analysis. The cutoff value of 16 gave the sensitivity and specificity of 88 and 100%, respectively. The area under the ROC curve was 0.9546.

ICT signals generated from all sera were quantified and converted to IV units (see Materials and Methods) (Fig. 2). The mean IV for vascular pythiosis patients was 67.2 U (range, 22.4 to 113.3 U), whereas that for cutaneous pythiosis patients was 17.7 U (range, 16.0 to 19.4 U), that for ocular pythiosis patients was 4.3 U (range, 0 to 7.4 U), that for blood donors was 3.8 U (range, 0 to 12.3 U), that for patients with other infectious diseases was 2.5 U (range, 0 to 11.9 U), that for thalassemic patients was 2.7 U (range, 0 to 5.9 U), and that for patients with autoimmune diseases or TAO was 2.1 U (range, 0 to 3.8 U). As an alternative to the determination of results by three independent laboratory personnel, the IV cutoff point was selected by a ROC analysis to differentiate between patients with and without human pythiosis. The ICT has very good discriminative power for identifying patients with human pythiosis, as shown by the large area (0.95) under the ROC curve (Fig. 3). The IV of 16.0 was selected as the cutoff value, because it gave the highest sensitivity (88%) and specificity (100%). For comparison, the IV cutoff value of 12.3 yielded a sensitivity of 88% and a specificity of 99% and the IV cutoff value of 19.4 yielded a sensitivity of 85% and a specificity of 100%.

DISCUSSION

The ID test has high specificity but poor sensitivity and requires a long turnaround time. Because early and accurate diagnosis would improve the clinical outcomes for patients with pythiosis, the ICT was developed to address this issue. Thirty-three pythiosis patient sera and 181 control sera from healthy blood donors and patients with thalassemia (a predisposing factor for pythiosis), various infectious diseases, autoimmune diseases, and TAO, which clinically mimics vascular pythiosis, were used for the comparison of ICT and ID performances. Pythiosis can be misdiagnosed as aspergillosis and zygomycosis because the causative agents have microscopic morphologies similar to that of *P. insidiosum* (16). Sera from

patients with aspergillosis, zygomycosis, and other endemic infectious diseases (such as melioidosis, HIV infection, and malaria) were tested for any background or cross-reactivity. All control sera tested negative by the ICT and ID, demonstrating 100% specificity for both tests.

The sensitivity of the ICT to detect anti-*P. insidiosum* IgG in all pythiosis sera was greater than that of ID (88% for the ICT; 61% for ID). Sera from all ocular pythiosis patients tested negative by both the ICT and ID. The failure to detect anti-*P. insidiosum* antibodies in these patients was due likely to poorly induced antibody responses to localized infections of the eye (6). Therefore, the serodiagnosis of ocular infection should be avoided, because of an expected high rate of false-negative results. When ocular pythiosis patient sera were excluded from the evaluation, the sensitivity of the ICT increased to 100% and that of ID increased to 69%. The ICT is a ready-to-use test, while ID is complicated by a need to prepare a fresh diffusion gel right before performing the test. The turnaround time of the ICT was remarkably shorter than that of ID (30 min for the ICT; ~24 h for ID). Therefore, the ICT shows better sensitivity and is more convenient than ID. It is suitable for the serodiagnosis of vascular and cutaneous pythiosis but not ocular pythiosis.

The two highest IVs among the controls (Fig. 2) were obtained for a blood donor (12.3 U) and a patient with penicilliosis (11.9 U). The ICT results for the control sera from these subjects were reported as negative by the readers, as no test signal was grossly detected. Among patients positive for pythiosis (Fig. 2), two cutaneous pythiosis patients had the lowest IVs (16.0 and 19.4 U). Low anti-*P. insidiosum* IgG levels in these cutaneous pythiosis patients were already expected because one patient had advanced HIV infection and a low CD4 cell count (52 cells/μl) and the other had an acute *P. insidiosum* infection with a few-day history of symptoms prior to hospital admission. Nevertheless, the sera were reported to be positive, as faint test lines were consistently and unambiguously detected by the three readers. These findings indicated that the ICT had good discriminating power for negative and weakly positive samples.

In conclusion, the in-house ICT had higher sensitivity and specificity, required a shorter turnaround time, and was a more user-friendly test than ID. In addition, the ICT is suitable for use at the bedside, as well as in remote hospitals where skilled personnel or diagnostic materials are lacking.

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REFERENCES

1. **Chaiprasert, A., S. Samerpitak, W. Wanachiwanawin, and P. Thasnakorn.** 1990. Induction of zoospore formation in Thai isolates of *Pythium insidiosum*. Mycoses **33:**317–323.
2. **Chetchotisakd, P., C. Pairojkul, O. Porntaveevudhi, B. Sathapatayavongs, P. Mairiang, K. Nuntirooj, B. Patjanasoontorn, O. T. Saew, A. K. Chaiprasert, and M. R. Haswell-Elkins.** 1992. Human pythiosis in Srinagarind Hospital: one year's experience. J. Med. Assoc. Thai. **75:**248–254.
3. **Imwidthaya, P.** 1994. Human pythiosis in Thailand. Postgrad. Med. J. **70:**558–560.
4. **Imwidthaya, P., and S. Srimuang.** 1989. Immunodiffusion test for diagnosing human pythiosis. Mycopathologia **106:**109–112.
5. **Kaufman, L.** 1998. Penicilliosis marneffei and pythiosis: emerging tropical diseases. Mycopathologia **143:**3–7.
6. **Krajaejun, T., M. Kunakorn, S. Niemhom, P. Chongtrakool, and R. Pracharktam.** 2002. Development and evaluation of an in-house enzyme-linked immunosorbent assay for early diagnosis and monitoring of human pythiosis. Clin. Diagn. Lab. Immunol. **9:**378–382.
7. **Krajaejun, T., M. Kunakorn, R. Pracharktam, P. Chongtrakool, B. Sathapatayavongs, A. Chaiprasert, N. Vanittanakom, A. Chindamporn, and P. Mootsikapun.** 2006. Identification of a novel 74-kilodalton immunodominant antigen of *Pythium insidiosum* recognized by sera from human patients with pythiosis. J. Clin. Microbiol. **44:**1674–1680.
8. **Krajaejun, T., R. Pracharktam, S. Wongwaisayawan, M. Rochanawutinon, M. Kunakorn, and S. Kunavisarut.** 2004. Ocular pythiosis: is it underdiagnosed? Am. J. Ophthalmol. **137:**370–372.
9. **Krajaejun, T., B. Sathapatayavongs, R. Pracharktam, P. Nitiyanant, P. Leelachaikul, W. Wanachiwanawin, A. Chaiprasert, P. Assanasen, M. Saipetch, P. Mootsikapun, P. Chetchotisakd, A. Lekhakula, W. Mitarnun, S. Kalnauwakul, K. Supparatpinyo, R. Chaiwarith, S. Chiewchanvit, N. Tananuvat, S. Srisiri, C. Suankratay, W. Kulwichit, M. Wongsaisuwan, and S. Somkaew.** 2006. Clinical and epidemiological analyses of human pythiosis in Thailand. Clin. Infect. Dis. **43:**569–576.
10. **Kwon-Chung, K. J.** 1994. Phylogenetic spectrum of fungi that are pathogenic to humans. Clin. Infect. Dis. **19:**S1–7.
11. **Mendoza, L., L. Ajello, and M. R. McGinnis.** 1996. Infection caused by the oomycetous pathogen *Pythium insidiosum*. J. Mycol. Med. **6:**151–164.
12. **Mendoza, L., F. Hernandez, and L. Ajello.** 1993. Life cycle of the human and animal oomycete pathogen *Pythium insidiosum*. J. Clin. Microbiol. **31:**2967–2973.
13. **Mendoza, L., L. Kaufman, W. Mandy, and R. Glass.** 1997. Serodiagnosis of human and animal pythiosis using an enzyme-linked immunosorbent assay. Clin. Diagn. Lab. Immunol. **4:**715–718.
14. **Mendoza, L., L. Kaufman, and P. G. Standard.** 1986. Immunodiffusion test for diagnosing and monitoring pythiosis in horses. J. Clin. Microbiol. **23:**813–816.
15. **Mendoza, L., V. Nicholson, and J. F. Prescott.** 1992. Immunoblot analysis of the humoral immune response to *Pythium insidiosum* in horses with pythiosis. J. Clin. Microbiol. **30:**2980–2983.
16. **Mendoza, L., S. H. Prasla, and L. Ajello.** 2004. Orbital pythiosis: a nonfungal disease mimicking orbital mycotic infections, with a retrospective review of the literature. Mycoses **47:**14–23.
17. **Mendoza, L., and J. Prendas.** 1988. A method to obtain rapid zoosporogenesis of *Pythium insidiosum*. Mycopathologia **104:**59–62.
18. **Pracharktam, R., P. Changtrakool, B. Sathapatayavongs, P. Jayanetra, and L. Ajello.** 1991. Immunodiffusion test for diagnosis and monitoring of human pythiosis insidiosi. J. Clin. Microbiol. **29:**2661–2662.
19. **Prasertwitayakij, N., W. Louthrenoo, N. Kasitanon, K. Thamprasert, and N. Vanittanakom.** 2003. Human pythiosis, a rare cause of arteritis: case report and literature review. Semin. Arthritis Rheum. **33:**204–214.
20. **Pupaibool, J., A. Chindamporn, K. Patarakul, C. Suankratay, W. Sindhuphak, and W. Kulwichit.** 2006. Human pythiosis. Emerg. Infect. Dis. **12:**517–518.
21. **Sathapatayavongs, B., P. Leelachaikul, R. Prachaktam, V. Atichartakarn, S. Sriphojanart, P. Trairatvorakul, S. Jirasiritham, S. Nontasut, C. Eurvilaichit, and T. Flegel.** 1989. Human pythiosis associated with thalassemia hemoglobinopathy syndrome. J. Infect. Dis. **159:**274–280.
22. **Tanphaichitra, D.** 1989. Tropical disease in the immunocompromised host: melioidosis and pythiosis. Rev. Infect. Dis. **11:**S1629–S1643.
23. **Thianprasit, M., A. Chaiprasert, and P. Imwidthaya.** 1996. Human pythiosis. Curr. Top. Med. Mycol. **7:**43–54.
24. **Thitithanyanont, A., L. Mendoza, A. Chuansumrit, R. Pracharktam, J. Laothamatas, B. Sathapatayavongs, S. Lolekha, and L. Ajello.** 1998. Use of an immunotherapeutic vaccine to treat a life-threatening human arteritic infection caused by *Pythium insidiosum*. Clin. Infect. Dis. **27:**1394–1400.
25. **Vanittanakom, N., J. Supabandhu, C. Khamwan, J. Praparattanapan, S. Thirach, N. Prasertwitayakij, W. Louthrenoo, S. Chiewchanvit, and N. Tananuvat.** 2004. Identification of emerging human-pathogenic *Pythium insidiosum* by serological and molecular assay-based methods. J. Clin. Microbiol. **42:**3970–3974.
26. **Wanachiwanawin, W., L. Mendoza, S. Visuthisakchai, P. Mutsikapan, B. Sathapatayavongs, A. Chaiprasert, P. Suwanagool, W. Manuskiatti, C. Ruangsetakit, and L. Ajello.** 2004. Efficacy of immunotherapy using antigens of *Pythium insidiosum* in the treatment of vascular pythiosis in humans. Vaccine **22:**3613–3621.
27. **Wanachiwanawin, W., M. Thianprasit, S. Fucharoen, A. Chaiprasert, N. Sudasna, N. Ayudhya, N. Sirithanaratkul, and A. Piankijagum.** 1993. Fatal arteritis due to *Pythium insidiosum* infection in patients with thalassaemia. Trans. R. Soc. Trop. Med. Hyg. **87:**296–298.

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Hemagglutination Test for Rapid Serodiagnosis of Human Pythiosis▽

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Human pythiosis is an emerging, life-threatening infectious disease, caused by the oomycete *Pythium insidiosum*. Thailand is an area where human pythiosis is endemic and the genetic blood disorder thalassemia is a predisposing factor. Patients with pythiosis present with arterial occlusions of the lower extremities, corneal ulcers, or chronic cutaneous infections. Diagnosis relies on time-consuming, relatively insensitive tests such as culture identification and immunodiffusion assay. Most patients undergo surgical removal of infected organs, and many die from the infection. Delayed diagnosis results in a poor prognosis. Here, we describe a hemagglutination (HA) test for rapid diagnosis of human pythiosis. Sheep red blood cells were coated with *P. insidiosum* protein extract and used in duplicated detection assays using serum samples from 33 patients with vascular ($n = 27$), cutaneous ($n = 2$), or ocular ($n = 4$) pythiosis and serum samples from 289 control patients with other infectious diseases ($n = 77$), with highly positive antinuclear antibody ($n = 5$), with thalassemia ($n = 21$), or with no known disorder (i.e., healthy blood donors) ($n = 186$). Based on receiver-operating characteristic analysis, a serum titer of 1:160 was selected as the cutoff point for the HA test. Serum samples that generated HA at the cutoff titer were read as positive, while samples that did not were read as negative. Positive results were obtained with the serum samples of all patients with vascular and cutaneous pythiosis and with two serum samples from the control group. Negative results were obtained with serum samples from all ocular pythiosis patients and the 287 remaining serum samples from the control group. Sensitivity and specificity of the HA were 88% and 99%, respectively. In conclusion, the HA test for detection of anti-*Pythium* antibodies is a simple, rapid, and reliable test for serodiagnosis of vascular and cutaneous pythiosis.

Pythiosis is can be a fatal infectious disease of humans and animals living in tropical and subtropical countries (2, 9, 15, 16, 18, 27, 30). The causative agent is the fungus-like organism *Pythium insidiosum*, which is a member of the family Pythiaceae, order Pythiales, class Oomycetes, and the phylum Pseudofungi in the kingdom Chromista (Stramenopila) (5, 9, 18, 30). Naturally, *P. insidiosum* inhabits swampy areas, where it is present in the form of mycelium or biflagellate zoospores (5, 19). The zoospore is an infective stage where it can swim, attach to, and penetrate host tissue, possibly leading to pathology (18, 19).

Although pythiosis in animals has been increasingly reported worldwide, most human pythiosis cases have been reported in Thailand, where it is considered to be endemic (8, 14, 16, 17, 26, 28, 30, 33). Thalassemia and agriculture-related careers are predisposing factors for human pythiosis (16, 17, 28). Clinical features of human pythiosis can be categorized into four forms as follows. (i) Vascular pythiosis (59% of reported cases) is an infection of the arteries leading to arterial occlusion and aneurysm. In advanced cases, many patients die, and since the main treatment is limb amputation, many patients become handicapped. (ii) Ocular pythiosis (33%) is an infection of the eyes, in which patients usually present with corneal ulcers or keratitis. Most of these patients undergo enucleation therapy to control the infection. (iii) Patients with cutaneous pythiosis (5%) present with granulomatous and ulcerative lesions confined to cutaneous and subcutaneous tissues. (iv) Disseminated pythiosis (3%) is an infection of other internal organs, such as the brain, sinuses, or gastrointestinal tract. The use of conventional antifungal drugs is ineffective in treatment of pythiosis because *Pythium* is only distantly related phylogenetically to fungi, and radical surgery is the main treatment option (16, 17, 29).

Delayed diagnosis leads to delayed treatment and a poorer prognosis in patients with pythiosis. Diagnosis by culture identification of *P. insidiosum* is time-consuming and laborious (3, 23). Serodiagnosis of pythiosis commonly relies on an immunodiffusion (ID) test. Although the ID test is highly specific, it has very poor sensitivity (11, 12, 21, 25). Subsequently, other diagnostic methods, such as an in-house enzyme-linked immunosorbent assay (ELISA), an immunochromatographic test (ICT), a Western blot assay, and a PCR assay, were developed and have good specificity and sensitivity (11–13, 20, 22, 32). However, the lack of diagnostic materials and special equipment needed for these tests limits their use, especially in rural areas where the disease is prevalent. Here, we describe a hemagglutination (HA) test to assist a rapid diagnosis of human pythiosis. The test is easy to perform, requires only routine laboratory equipment and could easily be adapted to a simple kit format.

MATERIALS AND METHODS

Serum samples. A total of 33 serum samples from patients with pythiosis (27 vascular, four ocular, and two cutaneous) were recruited for the assay evaluation. Clinical information was recorded for each pythiosis patient and included clinical features, duration of symptoms before the first medical visit, underlying diseases,

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TABLE 1. Clinical information of pythiosis patients from whom serum samples were obtained for the HA test evaluation[g]

Patient	Form of infection	Underlying disease[a]	Clinical presentation	Duration of symptom[b]	Diagnosis			HA titer (test result)[f]
					Histology[c]	Culture[d]	Serology[e]	
S1	Vascular	Thal	Claudication, ulcer	5 mo	(+)	N/A[g]	1 (+), 4 (+)	5,120 (+)
S2	Vascular	Thal	Claudication, ulcer	2 mo	(+)	(+)	1 (−), 4 (+)	1,280 (+)
S3	Vascular	PNH	Gangrenous ulcer	3 wk	N/A	(+)	1 (+), 4 (+)	1,280 (+)
S4	Vascular	Thal	Claudication, ulcer	1 mo	(+)	(+)	1 (+), 2 (+), 4 (+)	2,560 (+)
S5	Vascular	Thal	Claudication, ulcer	7 wk	(+)	(+)	1 (+), 2 (+), 4 (+)	1,280 (+)
S6	Vascular	Thal	Claudication, ulcer	5 mo	N/A	(+)	1 (−), 4 (+)	5,120 (+)
S7	Vascular	Thal	Claudication, ulcer	1 mo	(+)	N/A	1 (+), 4 (+)	20,480 (+)
S8	Vascular	Thal	Claudication, ulcer	3 mo	(+)	N/A	1 (+), 4 (+)	1,280 (+)
S9	Vascular	Thal	Claudication, ulcer	1 mo	(+)	(+)	1 (−), 4 (+)	320 (+)
S10	Vascular	Thal	Claudication, ulcer	10 days	(+)	(+)	1 (+), 4 (+)	5,120 (+)
S11	Vascular	Thal	Claudication, ulcer	3 mo	(+)	(+)	1 (+), 4 (+)	2,560 (+)
S12	Vascular	Thal	Gangrenous ulcer	12 mo	N/A	N/A	1 (+), 3 (+), 4 (+)	20,480 (+)
S13	Vascular	Thal	Claudication, ulcer	N/A	(+)	N/A	1 (+), 4 (+)	1,280 (+)
S14	Vascular	Thal	Claudication, ulcer	N/A	(+)	N/A	1 (−), 4 (+)	1,280 (+)
S15	Vascular	Thal	Claudication, ulcer	N/A	(+)	N/A	1 (−), 4 (+)	1,280 (+)
S16	Vascular	Thal	Claudication, ulcer	1 mo	(+)	N/A	1 (−), 4 (+)	160 (+)
S17	Vascular	N/A	Claudication, ulcer	N/A	N/A	N/A	1 (+), 4 (+)	1,280 (+)
S18	Vascular	N/A	Claudication, ulcer	N/A	N/A	N/A	1 (+), 4 (+)	1,280 (+)
S19	Vascular	Thal	Claudication, ulcer	N/A	(+)	(+)	1 (−), 4 (+)	320 (+)
S20	Vascular	Thal	Claudication, ulcer	3 mo	(+)	N/A	1 (+), 4 (+)	40,960 (+)
S21	Vascular	Thal	Claudication, ulcer	5 mo	(+)	N/A	1 (+), 4 (+)	10,240 (+)
S22	Vascular	Thal	Claudication, ulcer	N/A	(+)	N/A	1 (+), 4 (+)	640 (+)
S23	Vascular	N/A	Claudication, ulcer	N/A	N/A	N/A	1 (+), 3 (+), 4 (+)	10,240 (+)
S24	Vascular	N/A	Claudication, ulcer	N/A	N/A	N/A	1 (+), 3 (+), 4 (+)	1,280 (+)
S25	Vascular	N/A	Claudication, ulcer	N/A	N/A	N/A	1 (+), 3 (+), 4 (+)	10,240 (+)
S26	Vascular	N/A	Claudication, ulcer	N/A	N/A	N/A	1 (+), 3 (+), 4 (+)	2,560 (+)
S27	Vascular	N/A	Claudication, ulcer	N/A	N/A	N/A	1 (−), 3 (+), 4 (+)	160 (+)
S28	Cutaneous	Thal	Necrotizing cellulitis	6 days	N/A	(+)	1 (+), 4 (+)	320 (+)
S29	Cutaneous	HIV, ITP	Chronic ulcer	3 mo	N/A	(+)	1 (−), 3 (+), 4 (+)	640 (+)
S30	Ocular	N/A	Keratitis	10 days	(+)	(+)	1 (−), 4 (−)	80 (−)
S31	Ocular	N/A	Corneal ulcer	N/A	N/A	(+)	1 (−), 3 (−), 4 (−)	40 (−)
S32	Ocular	N/A	Corneal ulcer	N/A	N/A	(+)	1 (−), 3 (−), 4 (−)	40 (−)
S33	Ocular	N/A	Corneal ulcer	N/A	N/A	(+)	1 (−), 3 (−), 4 (−)	80 (−)

[a] Thal, thalassemia; PNH, paroxysmal nocturnal hemoglobinuria; HIV, human immunodeficiency virus; ITP, idiopathic thrombocytopenic purpura.
[b] Duration of symptom starting from when it was first recognized by a patient before seeking medical attention.
[c] (+), positive Grocott's methenamine silver staining for fungal elements compatible to *P. insidiosum* in infected tissue.
[d] (+), successful isolation of *P. insidiosum* by culture identification method.
[e] 1, ID test; 2, ELISA; 3, Western blotting; 4, ICT; (+), positive result; (−), negative result.
[f] HA titer represents reciprocal titer. (+), positive result; (−), negative result.
[g] N/A, data not available.

and method of diagnosis (Table 1). All pythiosis patients were diagnosed based on at least one of following criteria: (i) *P. insidiosum* isolated from infected tissue and confirmed by induction and identification of zoospores, or (ii) the presence of anti–*P. insidiosum* antibodies in blood samples; antibody detection was by at least one of the following well-established serodiagnostic tests: ID test, ELISA, Western blot analysis, or ICT (3, 11–13, 15–18, 20–23, 25, 32). Additional serum samples (n = 289) were collected as control samples that included (i) 186 randomly collected serum samples from healthy blood donors at the Blood Bank Division of Ramathibodi Hospital, (ii) 21 serum samples from healthy thalassemic patients without clinical evidence of pythiosis, (iii) five serum samples from patients with highly positive antinuclear antibody, and (iv) 77 serum samples from patients positive for other infectious diseases. The last group included 19 serum samples obtained from patients with proven cryptococcosis (n = 11), penicillosis (n = 7), or candidiasis (n = 1), as determined by criteria for invasive fungal diseases of the European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group (EORTC/MSG) (6). Of the remaining 58 serum samples, 20 were obtained from patients with aspergillosis (n = 4) or mucormycosis (n = 4) confirmed by culture identification, from patients that were fungal galactomannan antigen positive (n = 9), and from patients that were anti-*Histoplasma capsulatum* antibody positive (n = 3). However, information on host factors and clinical features and other mycological evidence for revalidation by EORTC/MSG criteria were missing for these 20 samples. The remaining 38 out of 77 serum samples comprised samples from cases with proven nonfungal infections according to established criteria (4, 34). These included samples that were positive for anti-human immunodeficiency virus antibody (n = 10), syphilis (n = 9), malaria (n = 5), mycoplasmosis (n = 4), toxoplasmosis (n = 2), leptospirosis (n = 2), melioidosis (n = 2), anti-*Entamoeba histolytica* antibody (n = 1), hepatitis A virus (n = 1), hepatitis B virus (n = 1), and hepatitis C virus (n = 1). One positive control serum sample was obtained from a rabbit immunized with *P. insidiosum* antigen. All serum samples were stored at −20°C until used.

Antigen preparation. The *P. insidiosum* strain CBS119452 isolated from a Thai patient with vascular pythiosis was used to prepare the antigen. The microorganism was subcultured on Sabouraud dextrose agar and incubated at 37°C for 3 days. Several small blocks of mycelium were transferred to Sabouraud dextrose broth and shaken (150 rpm) at 37°C for 9 days. Merthiolate was added to the culture at a final concentration of 0.02% (wt/vol). The culture was filtered through a Durapore membrane filter (0.22-μm pore size). Phenylmethylsulfonyl fluoride (0.1 mg/ml) and EDTA (0.3 mg/ml) were added to the culture filtrate broth before concentration to ~80-fold using an Amicron 8400 apparatus and an Amicon Ultra-15 centrifugal filter (10,000 nominal molecular weight limit; Millipore, Bedford, MA). The concentrated broth was referred to as culture filtrate antigen (CFA), and was measured for protein concentration by a spectrophotometer. CFA was stored at −20°C.

HA test. (i) Stabilization of sheep red blood cells. Preparation of sheep red cells (SRC) for the HA test was modified from the methods of Hirata and Brandriss (7) and Petchclai et al. (24). The SRC were packed by washing with normal saline and centrifuging at 5,000 rpm for 3 min three times. Then, 1.25 ml of 0.15 M phosphate-buffered saline (PBS) (pH 7.2) and 0.25 ml of 2.5% glu-

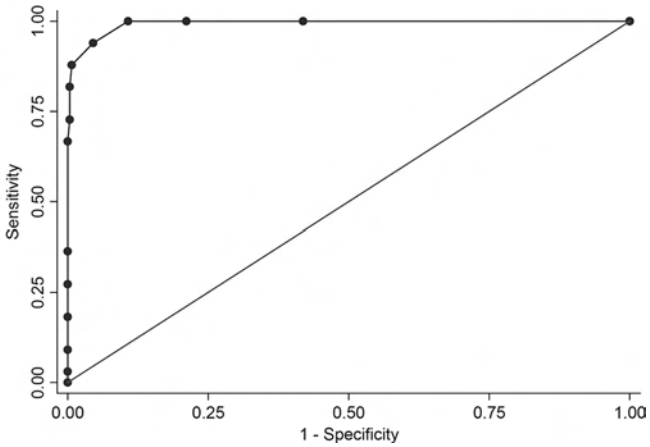

FIG. 1. ROC curve for the HA test. The area under the ROC curve is 0.9931.

taraldehyde in distilled water were added to 0.1 ml of the packed SRC, and then they were gently mixed by rotator at room temperature for 2 h. The SRC were washed with normal saline and centrifuged three times as described above. PBS with 0.1% sodium azide was added to the SRC to make a 10% glutaraldehyde-stabilized SRC suspension.

(ii) Preparation of *P. insidiosum* antigen-coated SRC. To coat SRC with *P. insidiosum* antigens, 0.1 ml of 2-mg/ml CFA and 1 ml of 0.1 M acetate buffer (pH 4) were sequentially added to 0.1 ml of 10% glutaraldehyde-stabilized SRC suspension, before mixing and incubating at 37°C for 30 min. The CFA-coated SRC were washed with normal saline and centrifuged three times (as described above) and resuspended in PBS with 0.1% bovine serum albumin and 0.1% sodium azide to make a 0.5% CFA-coated SRC suspension.

(iii) HA assay. To perform the HA test, 25 μl of 1:10 diluted serum was added to the first well of a 96-U-shaped-well microtiter plate. Then, the serum was diluted twofold from 1:10 to 1:20,480 by using a diluent (0.5% bovine serum albumin, 1% normal rabbit serum, and 0.1% sodium azide in PBS). Twenty-five microliters of the 0.5% CFA-coated SRC suspension was added to each well and mixed gently. The positive control well contained the CFA-coated SRC suspension mixed with a positive serum. The negative control wells contained CFA-coated SRC suspension and the diluent or a negative serum. Each sample was tested in duplicate. The plate was incubated for 1 h at room temperature. The presence of HA was read as a positive test result for *P. insidiosum*, whereas the absence of HA was read as a negative test result.

ID test. The ID test was modified from the original method of Pracharktam et al. (25). Briefly, agar gel diffusion was carried out in a 5-cm-diameter petri dish with 2% agar in Veronal buffer (0.9% [wt/vol] $C_8H_{11}N_2NaO_3$, 0.05% [wt/vol] NaN_3 [pH 8.6]). The CFA and a serum sample were each added to 4-mm-diameter wells that were set 4 mm apart from each other. The plates were incubated in a moist chamber at room temperature for 24 h. The presence of an identity precipitation line with the positive control serum was considered a positive test result for *P. insidiosum*.

Statistical analysis. Sensitivities, specificities, and accuracies were calculated from each cutoff titer of the HA test and displayed in a receiver-operating characteristic (ROC) curve using the Stata program, version 10.0 (StataCorp, TX).

RESULTS

The area under the ROC curve for the HA test results was 0.99 (Fig. 1). The best cutoff titer chosen for the HA test was 1:160 because it provided the highest accuracy (98.1%) (Table 2). At this cutoff titer, all 29 serum samples from vascular and cutaneous pythiosis patients, serum from one healthy blood donor, and serum from a patient with mycoplasmosis (i.e., two samples from the negative control group) gave positive test results, while all four serum samples from the ocular pythiosis patients and the remaining 287 serum samples from the negative control patients gave negative test results. Therefore, sensitivity and specificity of the HA test were 87.9% and 99.3%, respectively. Incubation time for the HA test was 1 h.

To calculate positive and negative predictive values of the HA test, prevalence of pythiosis, test sensitivity (88%), and test specificity (99%) were used as in the statistic equation described elsewhere (1). At Ramathibodi Hospital during the year 2008, the prevalences of pythiosis in all patients (n = 190,415) and in patients with thalassemia (a prominent predisposing factor for pythiosis) (n = 372) were 0.002% and 0.8%, respectively. The positive predictive value (PPV) and negative predictive value (NPV) of the HA test using the prevalence in all patients were 0.2% and 100.0%, respectively, while the PPV and NPV using the prevalence in thalassemic patients were 41.7% and 99.9%, respectively.

To test the precision of the HA test, one each of the positive control and negative control serum samples was repeatedly assayed 20 times in both within-run and between-run analyses. The highest HA titer obtained from each precision analysis was 1:640 for the positive control serum, and test were negative at all titers for the negative control serum. The CFA-coated SRC were stable for at least 6 months, since reactions of the positive control serum remained unchanged at the highest HA titer (1:640) over the interval (i.e., at 2 weeks, 1 month, 3 months, 5 months, and 6 months after the preparation of coated SRC).

For the ID test, only 20 serum samples from patients with vascular (n = 19) and cutaneous (n = 1) pythiosis gave positive results. All of the control serum samples and 13 serum samples from patients with vascular (n = 8), ocular (n = 4), and cutaneous (n = 1) pythiosis gave negative test results. Therefore, sensitivity, specificity, and accuracy of the ID test were 60.6%, 100.0%, and 96.0%, respectively. Incubation time for the ID test was ~24 h.

TABLE 2. Sensitivity, specificity, and accuracy of the HA test at various cutoff serum titers[d]

Cutoff point (reciprocal titer)	Sensitivity (%)[a]	Specificity (%)[b]	Accuracy (%)[c]
Undiluted	100.0	0.0	10.3
10	100.0	58.1	62.4
20	100.0	78.9	81.1
40	100.0	89.3	90.4
80	93.9	95.5	95.3
160	**87.9**	**99.3**	**98.1**
320	81.8	99.7	97.8
640	72.7	99.7	96.9
1,280	66.7	100.0	96.6
2,560	36.4	100.0	93.5
5,120	27.3	100.0	92.6
10,240	18.2	100.0	91.6
20,480	9.1	100.0	90.7
40,960	3.0	100.0	90.1

[a] Sensitivity = true positive/(false negative + true positive) × 100.
[b] Specificity = true negative/(false positive + true negative) × 100.
[c] Accuracy = (true positive + true negative)/(true positive + true negative + false positive + false negative) × 100.
[d] The titer of 1:160 was selected as the best cutoff point because it provided the highest test accuracy (98.1%). This cutoff titer is indicated in boldface type.

DISCUSSION

An HA test was developed to facilitate rapid serodiagnosis of human pythiosis. The large area under the ROC curve for the HA test (Fig. 1) indicated a very good discriminative power for identifying patients with human pythiosis. The serum dilution of 1:160 was selected as the cutoff titer for reading agglutination reactions, because it gave minimal nonspecific HA reactions (i.e., false-positive results) and high sensitivity and specificity (Table 2). The explanation for the two negative control serum samples that gave positive HA test results (i.e., from a healthy blood donor and a patient with mycoplasmosis) could be (i) the presence of anti-*P. insidiosum* antibodies in patients with subclinical pythiosis or with a previous *P. insidiosum* infection or (ii) nonspecific binding of antibodies to CFA or SRC.

As expected for the test, the calculated PPV was very low, and the NPV was very high, because pythiosis is a disease of very low prevalence. Thus, the results suggested that the diagnostic value of the HA test would be to rule out pythiosis. Using the HA test for patients with thalassemia gave a markedly higher PPV than that for general patients (i.e., 41.7% versus 0.2%, respectively). Based on the within-run and between-run analyses (see Results), the HA test showed good precision. The coated SRC had a long shelf life (≥6 months) when kept refrigerated.

Among the patients with well-developed pythiosis that gave positive HA results (Table 1), there were two cases (patients S28 and S29) with interesting and unusual clinical features. Patient S28 presented a history of 6 days of acute necrotizing cellulites in both legs. Initial diagnosis was in doubt because of a relatively short history of illness and presentation of unlikely symptoms for pythiosis. Another immunocompromised patient (S29) had a high human immunodeficiency virus load (418,000 copies/ml serum) and a low CD4 count (52 cells/μl). The HA test successfully detected anti-*P. insidiosum* antibodies in these cases, indicating good diagnostic efficiency.

A pitfall of the test evaluation was that it lacked serum samples from patients with early-stage pythiosis, during which typical clinical features may be absent or minimal. Such early-stage patients might have levels of anti-*P. insidiosum* antibody too low to be detected by the HA test, and this might lead to false-negative test results. In this regard, a prospective field trial of the HA test would be necessary. Similarly, use of the HA test in a seroprevalence study of thalassemic individuals predisposed to pythiosis would provide useful information on its possible application in screening people at risk and on the nature and epidemiology of the disease.

All serum samples from ocular pythiosis patients gave negative results by both the HA and ID tests, suggesting that patients with ocular pythiosis are seronegative. This phenomenon may due to the fact that the eye is classified as an immune privileged site that lacks normal immune functions such as T- and B-cell proliferation, generation and activation of NK cells, development of cytotoxic T cells, and immunoglobulin production in order to minimize immunopathology from local infections (10). Thus, due to very low antibody production, antibody detection is not generally used for diagnosis of ocular infections such as mycoses (31), and our results agree, showing that serological diagnosis of ocular pythiosis is not feasible due to the high likelihood of false-negative results.

When the HA test was compared to the ID test now commonly used for serodiagnosis of pythiosis, the specificity and the accuracy of the HA test (99.3% and 98.1%, respectively) and those of the ID test (100% and 96%, respectively) were similar. However, the sensitivity of the HA test (88%) was markedly higher than that of the ID test (61%). The ID test gave false-negative test results for nine proven cases of vascular and cutaneous pythiosis, while the HA test correctly detected these cases. Our work confirmed the results of previous studies showing that the ID test has poor sensitivity (11, 12). The assay turnaround time for the HA test (1 h) was significantly shorter than that for the ID test (24 h).

Animal pythiosis has been increasingly reported worldwide (9, 18, 27). Although Thailand is an area where human pythiosis is endemic (16), there have been no reports of animal pythiosis from this country. It may be that this is due to lack of a sensitive and specific test to facilitate its diagnosis rather than lack of its occurrence. Our HA test for efficient detection of anti-*Pythium* antibody in human patient sera does not require a host species-specific conjugate antibody as do other highly sensitive tests, such as ELISA, ICT, and Western blot analysis (11–13, 20, 22). Thus, we have shown that the HA test detected anti-*Pythium* antibody in the serum of the rabbit immunized with *P. insidiosum* antigen, and this suggests that it could be used to conveniently detect anti-*Pythium* antibody in the sera of other animals as well. As such, the HA test could be regarded as a sensitive and specific test for rapid serodiagnosis of pythiosis in both humans and animals.

Since the HA test had good precision, it was used for antibody tests during the follow-up period after surgical treatment for pythiosis in two patients with vascular pythiosis. Gradual decreases of the antibody titer occurred in both patients (dropping from 1:5,120 to 1:80 in one case, and from 1:640 to 1:80 in the other case) in a manner that correlated well with their clinical improvement after surgery (data not shown). The antibody titer started to decrease within 1 month after leg amputation. These preliminary results indicate that the HA test may be a useful tool for monitoring treatment success in human pythiosis. A study of a larger group of pythiosis patients would be required before this could be confirmed.

In conclusion, a reliable HA test for serodiagnosis of vascular and cutaneous pythiosis was developed, but it was not suitable for diagnosis of ocular pythiosis due to a high likelihood of false-negative results. Higher test sensitivity and shorter turnaround time were advantages of the HA test over the commonly used ID test. The HA test is easy to perform, and it is suitable for diagnosis of pythiosis in hospitals in rural areas where the disease is prevalent.

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REFERENCES

1. **Altman, D. G., and J. M. Bland.** 1994. Diagnostic tests 2: predictive values. BMJ **309:**102.
2. **Bosco Sde, M., E. Bagagli, J. P. Araújo, Jr., J. M. Candeias, M. F. de Franco, M. E. Alencar Marques, L. Mendoza, R. P. de Camargo, and S. Alencar Marques.** 2005. Human pythiosis, Brazil. Emerg. Infect. Dis. **11:**715–718.
3. **Chaiprasert, A., S. Samerpitak, W. Wanachiwanawin, and P. Thasnakorn.** 1990. Induction of zoospore formation in Thai isolates of *Pythium insidiosum*. Mycoses **33:**317–323.
4. **Cheng, A. C., and B. J. Currie.** 2005. Melioidosis: epidemiology, pathophysiology, and management. Clin. Microbiol. Rev. **18:**383–416.
5. **De Cock, A. W., L. Mendoza, A. A. Padhye, L. Ajello, and L. Kaufman.** 1987. *Pythium insidiosum* sp. nov., the etiologic agent of pythiosis. J. Clin. Microbiol. **25:**344–349.
6. **De Pauw, B., T. J. Walsh, J. P. Donnelly, D. A. Stevens, J. E. Edwards, T. Calandra, P. G. Pappas, J. Maertens, O. Lortholary, C. A. Kauffman, D. W. Denning, T. F. Patterson, G. Maschmeyer, J. Bille, W. E. Dismukes, R. Herbrecht, W. W. Hope, C. C. Kibbler, B. J. Kullberg, K. A. Marr, P. Muñoz, F. C. Odds, J. R. Perfect, A. Restrepo, M. Ruhnke, B. H. Segal, J. D. Sobel, T. C. Sorrell, C. Viscoli, J. R. Wingard, T. Zaoutis, J. E. Bennett, et al.** 2008. Revised definitions of invasive fungal disease from the European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group (EORTC/MSG) Consensus Group. Clin. Infect. Dis. **46:**1813–1821.
7. **Hirata, A. A., and M. W. Brandriss.** 1968. Passive hemagglutination procedures for protein and polysaccharide antigens erythrocytes stabilized by aldehydes. J. Immunol. **100:**641–646.
8. **Imwidthaya, P.** 1994. Human pythiosis in Thailand. Postgrad. Med. J. **70:** 558–560.
9. **Kaufman, L.** 1998. Penicilliosis marneffei and pythiosis: emerging tropical diseases. Mycopathologia **143:**3–7.
10. **Koevary, S.** 2000. Ocular immune privilege: a review. Clin. Eye Vis. Care **12:**97–106.
11. **Krajaejun, T., S. Imkhieo, A. Intaramat, and K. Ratanabanangkoon.** 2009. Development of an immunochromatographic test for rapid serodiagnosis of human pythiosis. Clin. Vaccine Immunol. **16:**506–509.
12. **Krajaejun, T., M. Kunakorn, S. Niemhom, P. Chongtrakool, and R. Pracharktam.** 2002. Development and evaluation of an in-house enzyme-linked immunosorbent assay for early diagnosis and monitoring of human pythiosis. Clin. Diagn. Lab. Immunol. **9:**378–382.
13. **Krajaejun, T., M. Kunakorn, R. Pracharktam, P. Chongtrakool, B. Sathapatayavongs, A. Chaiprasert, N. Vanittanakom, A. Chindamporn, and P. Mootsikapun.** 2006. Identification of a novel 74-kilodalton immunodominant antigen of *Pythium insidiosum* recognized by sera from human patients with pythiosis. J. Clin. Microbiol. **44:**1674–1680.
14. **Krajaejun, T., R. Pracharktam, S. Wongwaisayawan, M. Rochanawutinon, M. Kunakorn, and S. Kunavisarut.** 2004. Ocular pythiosis: is it underdiagnosed? Am. J. Ophthalmol. **137:**370–372.
15. **Krajaejun, T., B. Sathapatayavongs, A. Chaiprasert, and S. Srimuang.** 2008. Do you know human pythiosis? J. Infect. Dis. Antimicrob. Agents **25:**45–51.
16. **Krajaejun, T., B. Sathapatayavongs, R. Pracharktam, P. Nitiyanant, P. Leelachaikul, W. Wanachiwanawin, A. Chaiprasert, P. Assanasen, M. Saipetch, P. Mootsikapun, P. Chetchotisakd, A. Lekhakula, W. Mitarnun, S. Kalnauwakul, K. Supparatpinyo, R. Chaiwarith, S. Chiewchanvit, N. Tananuvat, S. Srisiri, C. Suankratay, W. Kulwichit, M. Wongsaisuwan, and S. Somkaew.** 2006. Clinical and epidemiological analyses of human pythiosis in Thailand. Clin. Infect. Dis. **43:**569–576.
17. **Laohapensang, K., K. Rerkasem, J. Supabandhu, and N. Vanittanakom.** 2005. Necrotizing arteritis due to emerging *Pythium insidiosum* infection in patients with thalassemia: rapid diagnosis with PCR and serological test-case reports. Intern. J. Angiol. **14:**123–128.
18. **Mendoza, L., L. Ajello, and M. R. McGinnis.** 1996. Infection caused by the oomycetous pathogen *Pythium insidiosum*. J. Mycol. Med. **6:**151–164.
19. **Mendoza, L., F. Hernandez, and L. Ajello.** 1993. Life cycle of the human and animal oomycete pathogen *Pythium insidiosum*. J. Clin. Microbiol. **31:**2967–2973.
20. **Mendoza, L., L. Kaufman, W. Mandy, and R. Glass.** 1997. Serodiagnosis of human and animal pythiosis using an enzyme-linked immunosorbent assay. Clin. Diagn. Lab. Immunol. **4:**715–718.
21. **Mendoza, L., L. Kaufman, and P. G. Standard.** 1986. Immunodiffusion test for diagnosing and monitoring pythiosis in horses. J. Clin. Microbiol. **23:**813–816.
22. **Mendoza, L., V. Nicholson, and J. F. Prescott.** 1992. Immunoblot analysis of the humoral immune response to *Pythium insidiosum* in horses with pythiosis. J. Clin. Microbiol. **30:**2980–2983.
23. **Mendoza, L., and J. Prendas.** 1988. A method to obtain rapid zoosporogenesis of *Pythium insidiosum*. Mycopathologia **104:**59–62.
24. **Petchclai, B., R. Ausavarungnirun, and S. Manatsathit.** 1987. Passive hemagglutination test for enteric fever. J. Clin. Microbiol. **25:**138–141.
25. **Pracharktam, R., P. Changtrakool, B. Sathapatayavongs, P. Jayanetra, and L. Ajello.** 1991. Immunodiffusion test for diagnosis and monitoring of human pythiosis insidiosi. J. Clin. Microbiol. **29:**2661–2662.
26. **Pupaibool, J., A. Chindamporn, K. Patarakul, C. Suankratay, W. Sindhuphak, and W. Kulwichit.** 2006. Human pythiosis. Emerg. Infect. Dis. **12:** 517–518.
27. **Rivierre, C., C. Laprie, O. Guiard-Marigny, P. Bergeaud, M. Berthelemy, and J. Guillot.** 2005. Pythiosis in Africa. Emerg. Infect. Dis. **11:**479–481.
28. **Sathapatayavongs, B., P. Leelachaikul, R. Prachaktam, V. Atichartakarn, S. Sriphojanart, P. Trairatvorakul, S. Jirasiritham, S. Nontasut, C. Eurvilaichit, and T. Flegel.** 1989. Human pythiosis associated with thalassemia hemoglobinopathy syndrome. J. Infect. Dis. **159:**274–280.
29. **Schlosser, E., and D. Gottlieb.** 1966. Sterols and the sensitivity of *Pythium* species to filipin. J. Bacteriol. **91:**1080–1084.
30. **Thianprasit, M., A. Chaiprasert, and P. Imwidthaya.** 1996. Human pythiosis. Curr. Top. Med. Mycol. **7:**43–54.
31. **Thomas, P. A.** 2003. Current perspectives on ophthalmic mycoses. Clin. Microbiol. Rev. **16:**730–797.
32. **Vanittanakom, N., J. Supabandhu, C., Khamwan, J. Praparattanapan, S. Thirach, N. Prasertwitayakij, W. Louthrenoo, S. Chiewchanvit, and N. Tananuvat.** 2004. Identification of emerging human-pathogenic *Pythium insidiosum* by serological and molecular assay-based methods. J. Clin. Microbiol. **42:**3970–3974.
33. **Wanachiwanawin, W., L. Mendoza, S. Visuthisakchai, P. Mutsikapan, B. Sathapatayavongs, A. Chaiprasert, P. Suwanagool, W. Manuskiatti, C. Ruangsetakit, and L. Ajello.** 2004. Efficacy of immunotherapy using antigens of *Pythium insidiosum* in the treatment of vascular pythiosis in humans. Vaccine **22:**3613–3621.
34. **Woods, G. L., and J. B. Henry.** 2001. Medical microbiology, p. 1065–1066, 1084, 1134–1135, 1206, 1212, and 1213. *In* J. B. Henry (ed.), Clinical diagnosis and management by laboratory methods, 20th ed. W.B. Saunders Company, Philadelphia, PA.

โรค Human Pythiosis

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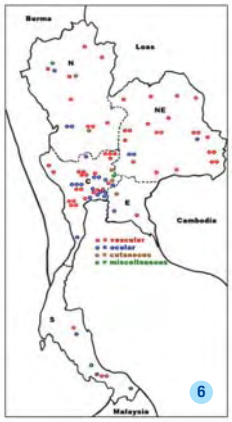
โรค pythiosis เป็นโรคติดเชื้ออุบัติใหม่ (emerging infectious disease) เกิดจากเชื้อ *Pythium insidiosum* (Figure 1, 2, 3,) ซึ่งเป็นเชื้อในกลุ่ม oomycetes และจัดอยู่ใน Kingdom Chromista/Straminopila โรคนี้มีความรุนแรงถึงขั้นพิการหรือเสียชีวิต พบได้ทั้งในคนและสัตว์ เช่น สุนัข แมว ม้า และวัว ที่อาศัยอยู่ในประเทศเขตร้อน ในธรรมชาติ เชื้อ *P. insidiosum* อาศัยอยู่ในน้ำหรือที่ชื้นแฉะ ในรูปของสายรา (Figure 2) ถึงแม้เชื้อ *P. insidiosum* นี้มีลักษณะทางกายภาพที่เหมือนเชื้อรา แต่เมื่อมีผู้วิเคราะห์ลักษณะทาง physiology และ genetics จึงทำให้ทราบว่าเชื้อนี้ไม่ถือเป็นเชื้อในกลุ่มรา ในภาวะแวดล้อมที่เหมาะสม เชื้อ *P. insidiosum* สามารถสร้าง zoospore (Figure 2) ซึ่งมี flagella ช่วยในการว่ายน้ำ Zoospore เป็นส่วนสำคัญของกระบวนการติดเชื้อ เมื่อ zoospore ว่ายน้ำมาสัมผัสกับผิวหนังหรือเยื่อบุต่าง ๆ ของคนหรือสัตว์ จะงอกเป็นสายรา (hyphae) ชอนไชไปสู่เนื้อเยื่อที่อยู่ลึกลงไป และก่อให้เกิดพยาธิสภาพ

โรค pythiosis ในคนนั้น พบครั้งแรกในประเทศไทย เมื่อ พ.ศ. 2528 หลังจากนั้นมีรายงานการพบผู้ป่วยเพิ่มขึ้นเรื่อย ๆ ส่วนใหญ่เป็นผู้ป่วยจากประเทศไทย จึงถือได้ว่าประเทศไทยเป็น endemic area ของโรค pythiosis ในคน แต่เนื่องจากบุคลากรทางการแพทย์ส่วนใหญ่ยังไม่รู้จักคุ้นเคยกับโรค และข้อมูลของโรคก็มีอยู่น้อย จึงได้มีการรวบรวมข้อมูลผู้ป่วยโรค pythiosis ที่วินิจฉัยระหว่าง พ.ศ. 2528-2546 จาก 9 คณะแพทยศาสตร์และโรงพยาบาลทั่วประเทศ มาวิเคราะห์ทางคลินิกและทางระบาดวิทยา (Krajaejun et al., Clin Infect Dis 2006; 43:569-76) เพื่อให้เข้าใจโรคในทางคลินิกมากขึ้น และเพื่อเป็นข้อมูลพื้นฐานสำหรับการวิจัยแง่มุมอื่น ในการศึกษานี้ พบผู้ป่วยจำนวน 102 ราย โดย 40% ของผู้ป่วย ได้รับการวินิจฉัยในช่วง 3-4 ปีสุดท้ายของการศึกษา แสดงว่าแพทย์เริ่มรู้จักโรคนี้มากขึ้น จึงมีแนวโน้มที่จะพบโรค pythiosis มากขึ้นในอนาคต ลักษณะทางคลินิกของโรค pythiosis สามารถแบ่งได้เป็น 4 กลุ่ม คือ

(1) ติดเชื้อที่ผิวหนัง *(Cutaneous/Subcutaneous pythiosis):* พบ 5% ของผู้ป่วยทั้งหมด ผู้ป่วยมาด้วยแผลเรื้อรัง ฝี-หนอง หรือการอักเสบของชั้นผิวหนังและใต้ผิวหนังบริเวณใบหน้า แขน หรือขา ส่วนใหญ่สามารถรักษาได้ด้วย saturated solution potassium iodide.

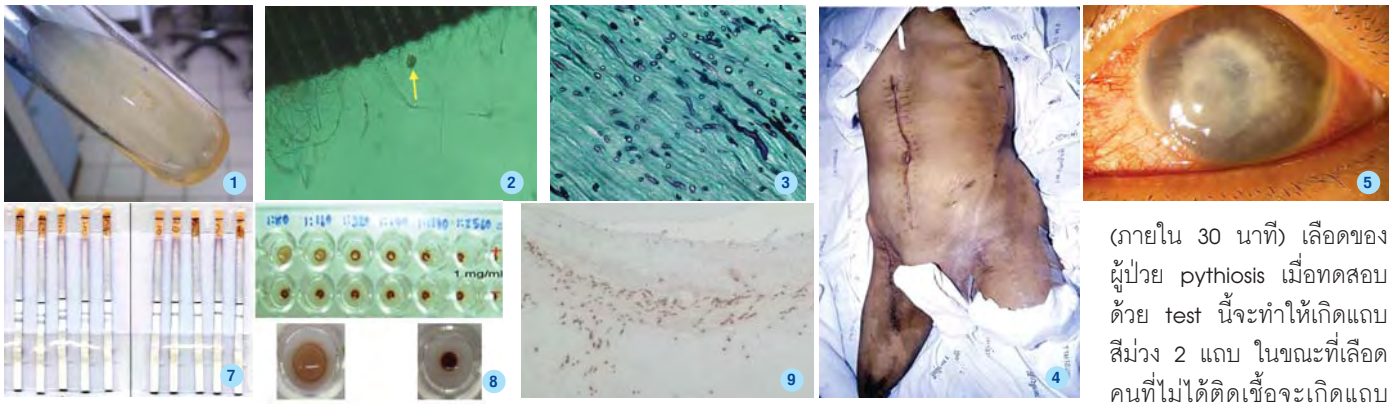
(2) ติดเชื้อที่เส้นเลือดแดง *(Vascular pythiosis):* พบ 59% ของผู้ป่วยทั้งหมด ผู้ป่วยมาด้วย claudication หรือ gangrene ที่ขา เพราะมีการติดเชื้อที่ผนังหลอดเลือดแดงจนเกิดการอุดตัน (Figure 4) ส่วนใหญ่ (~80% ของผู้ป่วย) รักษาโดยการตัดขาข้างที่ติดเชื้อทิ้ง ในรายที่มีการติดเชื้อลุกลามมากก็อาจเสียชีวิตได้จากการแตกของหลอดเลือดแดงใหญ่ (~40% ของผู้ป่วย)

(3) ติดเชื้อที่ตา *(Ocular pythiosis):* พบ 33% ของผู้ป่วยทั้งหมด ผู้ป่วยมักจะมาด้วยแผลที่กระจกตา ประมาณ 2-3 สัปดาห์ (Figure 5) การติดเชื้อมักลุกลาม ต้องรักษาโดยการผ่าตัดนำลูกตาข้างที่ติดเชื้อออก (~80% ของผู้ป่วย)



(4) ติดเชื้อบริเวณอื่น *(Disseminated pythiosis):* พบ 3% ของผู้ป่วยทั้งหมด ผู้ป่วยในกลุ่มนี้พบติดเชื้อที่เนื้อสมองที่โพรงจมูกและไซนัส หรือที่กระเพาะและลำไส้ ผู้ป่วยทั้งหมดในกลุ่มนี้เสียชีวิต

จากข้อมูลทางระบาดวิทยาของโรค human pythiosis ในประเทศไทย (Figure 6) พบมีผู้ป่วยกระจายอยู่ทั่วประเทศ จึงบ่งชี้ว่ามีการกระจายของเชื้อ *P. insidiosum* อยู่ทั่วประเทศ เมื่อดูจากลักษณะภูมิศาสตร์แล้ว เชื้อ *P. insidiosum* ก็น่าจะมีการกระจายที่ประเทศเพื่อนบ้านด้วย (ที่ผ่านมามีรายงานผู้ป่วยชาวมาเลเซียที่ติดเชื้อ *P. insidiosum* ที่ตามาแล้ว แต่จากประเทศเพื่อนบ้านอื่น ยังไม่มีการรายงานการพบผู้ป่วยแต่อย่างใด) นอกจากนี้ ยังพบด้วยว่ามีปัจจัยบางประการที่สัมพันธ์กับผู้ป่วยโรค pythiosis เช่น การทำอาชีพเกษตรกรรม (พบ 75% ของผู้ป่วย) ซึ่งเป็นอาชีพหลักของคนไทย, ช่วงอายุ 20-60 ปี (พบ 86% ของผู้ป่วย) ซึ่งเป็นช่วงวัยทำงาน และเป็นเพศชาย (พบ 71% ของผู้ป่วย) เป็นที่น่าสนใจว่า ผู้ป่วยที่มีรอยโรคที่เส้นเลือด ที่ผิวหนัง และที่อื่น ๆ ยกเว้นที่ตา ทุกคนป่วยเป็นโรคเลือดด้วย และโรคเลือดที่พบมากที่สุด คือ โรค thalassemia (85%) ซึ่งเป็นโรคทางพันธุกรรมที่พบบ่อยมากในประเทศไทย ส่วนผู้ป่วยที่มีรอยโรคที่ตานั้น พบว่าส่วนใหญ่ (84%) ไม่พบมีโรคประจำตัวใด ๆ ข้อมูลนี้จึงบ่งชี้ว่ามีปัจจัยบางประการที่ส่งเสริมให้ผู้ป่วยโรคเลือดติดเชื้อ *P. insidiosum* จนเกิดพยาธิสภาพ อย่างไรก็ตาม ผู้ที่มีสุขภาพแข็งแรง ไม่มีโรคประจำตัว ก็สามารถติดเชื้อ *P. insidiosum* ได้ การติดเชื้อเชื่อว่าเกิดจากการสัมผัสเชื้อ



(ภายใน 30 นาที) เลือดของผู้ป่วย pythiosis เมื่อทดสอบด้วย test นี้จะทำให้เกิดแถบสีม่วง 2 แถบ ในขณะที่เลือดคนที่ไม่ได้ติดเชื้อจะเกิดแถบสีม่วงเพียงแถบเดียว (Figure 7)

โดยตรงในแหล่งที่อยู่อาศัยของเชื้อ เช่น แหล่งน้ำทางเกษตรกรรม ทุ่งนา เป็นต้น

ปัญหาสำคัญของโรค pythiosis คือ มีอัตราทุพพลภาพและอัตราการตายที่สูง เนื่องจาก (1) บุคลากรทางการแพทย์ไม่รู้จักโรค (2) ขาด diagnostic test ที่ให้ผลรวดเร็ว ง่ายต่อการใช้ และมี sensitivity/specificity ที่ดี เพื่อใช้ในชนบทห่างไกลที่มีโอกาสพบผู้ป่วยได้มาก (3) การรักษาทางยาที่มีอยู่ในปัจจุบันใช้ไม่ได้ผล และวัคซีนที่ใช้รักษาโรคมีประสิทธิภาพต่ำ การรักษาหลักจึงต้องใช้การผ่าตัดเนื้อเยื่อหรืออวัยวะที่ติดเชื้อออกให้หมดซึ่งทำให้เกิดความพิการ บางครั้งถึงแม้จะทำการรักษาอย่างดีที่สุดแล้ว ก็ไม่สามารถรักษาชีวิตผู้ป่วยได้ เพราะเชื้อแพร่กระจายไปไกลแล้ว

สำหรับปัญหาที่บุคลากรไม่รู้จักคุ้นเคยกับโรคนั้น ปัจจุบันได้มีการเผยแพร่ให้มีการรู้จักโรคนี้มากขึ้นในหลาย ๆ ทาง เช่น การสอนนักศึกษาและแพทย์ให้รู้จักโรคนี้ในโรงเรียนแพทย์, การนำเสนอข้อมูลและงานวิจัยใหม่ ๆ ในวารสารและในที่ประชุมทั้งภายในประเทศและต่างประเทศ, การเผยแพร่ความรู้ของโรคนี้ผ่านทางรายการโทรทัศน์และหนังสือพิมพ์ ทั้งหมดนี้น่าจะทำให้บุคลากรทางการแพทย์ได้รู้จักโรคนี้มากขึ้น ทำให้เมื่อแพทย์พบผู้ป่วยที่มีอาการเข้าได้กับโรค human pythiosis ก็จะได้นึกถึงโรคนี้ไว้ด้วย แทนที่จะสงสัยว่าผู้ป่วยเป็นโรคติดเชื้อชนิดอื่น ๆ ที่มีลักษณะคล้ายกันเท่านั้น ทั้งนี้ จะส่งผลให้ผู้ป่วยได้รับการตรวจวินิจฉัยและรักษาได้ถูกต้องและรวดเร็วขึ้น

สำหรับปัญหาการวินิจฉัยและการรักษานั้น เนื่องจากมีการพบผู้ป่วยโรค pythiosis รายใหม่เพิ่มขึ้นเรื่อย ๆ โดยเฉพาะในประเทศไทย จึงมีความจำเป็นรีบด่วนในการวิจัยและพัฒนาวิธีการวินิจฉัยและรักษาใหม่ ๆ ที่ดีขึ้น เพื่อลดอัตราทุพพลภาพและอัตราการตายจากโรค นอกเหนือจากนี้การทำวิจัยพื้นฐานโรค pythiosis อย่างต่อเนื่องก็มีความจำเป็น เพราะเป็นการพัฒนาองค์ความรู้ใหม่ที่เป็นพื้นฐานต่อการพัฒนา ให้สามารถพึ่งตนเองทางด้านวิชาการได้ ในการแก้ปัญหาสำคัญของโรค human pythiosis ข้างต้น จึงมีนักวิจัยสนใจทำการศึกษาโรคและเชื้อก่อโรคนี้มากขึ้น สำหรับงานวิจัยที่ข้าพเจ้าและคณะกำลังมุ่งเน้นในขณะนี้ คือ

(1) <u>ด้านการวินิจฉัย</u> มีการพัฒนา rapid diagnostic tests ที่มีประสิทธิภาพมากขึ้น ใช้ง่าย และสามารถนำไปใช้ในท้องที่ห่างไกลได้ เช่น

(a) Immunochrmoatography test ซึ่งเป็นความร่วมมือกับ ศ.ดร.กวีรัตนบรรณางกูร และคณะ จากห้องปฏิบัติการอิมมูโนวิทยา สถาบันวิจัยจุฬาภรณ์ หลักการของ Test นี้จะเหมือนกับชุดทดสอบการตรวจการตั้งครรภ์จากปัสสาวะ ซึ่งมีความง่ายในการใช้ และสามารถอ่านผลได้รวดเร็ว

(b) Hemagglutination test อาศัยหลักการเกาะกลุ่มของเม็ดเลือดแดงแกะที่เคลือบผิวด้วยโปรตีนที่สกัดได้จากเชื้อ *P. insidiosum* สามารถใช้งานได้ง่าย และสามารถอ่านผลได้รวดเร็ว (ภายใน 60 นาที) เลือดของผู้ป่วย pythiosis เมื่อทดสอบด้วย test นี้จะทำให้เกิดการเกาะกลุ่มของเม็ดเลือดแกะ ในขณะที่เลือดคนที่ไม่ได้ติดเชื้อจะไม่เกิดการเกาะกลุ่มดังกล่าว (Figure 8) นอกจากนี้ หากสงสัยการติดเชื้อ *P. insidiosum* ในสัตว์ก็สามารถใช้ Test นี้ในการตรวจได้ด้วย งานวิจัยนี้เป็นงานวิจัยของแพทย์ประจำบ้านพยาธิวิทยา คณะแพทยศาสตร์ โรงพยาบาลรามาธิบดี ซึ่งดำเนินการโดย พญ. ธันยาสิริ จินดายก และคณะ

(c) Immunohistochemical staining Test นี้จะช่วยในการตรวจเนื้อเยื่อทางพยาธิวิทยา โดยการย้อมเนื้อเยื่อนั้นด้วย serum จากกระต่ายที่สร้าง antibody ต่อเชื้อ *P. insidiosum* แล้วใช้วิธีทางเคมีเพื่อทำให้เกิดสีที่ตัวเชื้อ ดังนั้น จะสามารถมองเห็นเชื้อในเนื้อเยื่อที่ส่งตรวจนั้นได้ชัดเจน และมีความจำเพาะสูง ซึ่งต่างจากการย้อมสีทางพยาธิวิทยาทั่วไปที่ไม่มีความจำเพาะ วิธีนี้จะทำให้พยาธิแพทย์สามารถให้การวินิจฉัยได้ถูกต้องแม่นยำขึ้นในการแยกการติดเชื้อ *P. insidiosum* ออกจากการติดเชื้อราชนิดอื่น ๆ ที่มีลักษณะคล้ายกัน เช่น *Aspergillus, Zygomycetes.* งานวิจัยนี้เป็นความร่วมมือกับ ผศ.นพ. นพดล ลาภเจริญทรัพย์ และคณะจากภาควิชาพยาธิวิทยา โรงพยาบาลรามาธิบดี

(2) <u>ด้านการรักษา</u> มีแนวคิดหลายอย่างที่จะพัฒนาการรักษาโรคนี้ให้ดีขึ้น เช่น การทดสอบความไวของยาต้านเชื้อรากลุ่มใหม่ การหาและสกัดสารยับยั้งเชื้อ *P. insidiosum* จากเชื้อราในธรรมชาติ, การทดสอบว่าอุณหภูมิมีผลต่อการเจริญเติบโตของเชื้อได้หรือไม่อย่างไร เพื่อที่จะนำมาใช้ประกอบการรักษา และการพัฒนา vaccine สำหรับโรค human pythiosis โดยใช้ recombinant protein ที่กระตุ้นภูมิคุ้มกันของร่างกายได้ดี ทั้งหมดอยู่ระหว่างการดำเนินการวิจัยโดยความร่วมมือกันของนักวิจัยในมหาวิทยาลัยมหิดล

โดยสรุป: โรค human pythiosis เป็นโรคประจำถิ่นของประเทศไทยที่มีความรุนแรงมาก การวินิจฉัยและการรักษาที่ถูกต้องรวดเร็ว จะสามารถช่วยลดอัตราการทุพพลภาพ และอัตราการตายของผู้ป่วยได้ จากความร่วมมือของนักวิจัยหลายท่าน ทำให้ขณะนี้เรามีชุดทดสอบที่มีประสิทธิภาพมากขึ้น ใช้งานได้ง่าย และสามารถอ่านผลได้รวดเร็ว ส่วนในด้านการรักษานั้น นักวิจัยก็กำลังศึกษาวิจัยกันอย่างเต็มที่เพื่อหาทางรักษาโรค human pythiosis ที่ดีขึ้น โดยหวังว่าในอนาคตอันใกล้เราจะมียา หรือ vaccine ที่ใช้รักษาโรคให้หายขาดได้ โดยผู้ป่วยปราศจากความพิการ

Go

เมนูข่าวหลัก

หน้าข่าวหลัก
ข่าวหน้า1
บทนำ
เศรษฐกิจ
ต่างประเทศ
การศึกษา
บันเทิง
กีฬา
กระบวนการยุติธรรม
ชีวิตคุณภาพ
ภูมิภาค
ผู้หญิง-ครอบครัว
สังคม
คอลัมน์
กระแสทรรศน์
ประชาชื่น
เลือกตั้ง
เศรษฐกิจต่างประเทศ
เศรษฐทรรศน์
การ์ตูน
ePeople

เลือกวันที่ย้อนหลัง

พื้นที่ผู้สนับสนุน

รายละเอียดข่าว

วันที่ 12 ธันวาคม พ.ศ. 2550 ปีที่ 30 ฉบับที่ 10868

โรคฮิวแมน พิธิโอซิส (Human pythiosis)

คอลัมน์ ส่องโรค ไขสุขภาพ

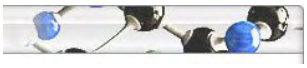
เป็นโรคติดเชื้อชนิดพิเธียม อินซิดิโอซั่ม (Pythium insidiosum) หรือที่เรียกว่า เชื้อราน้ำ พบมากในแหล่งน้ำทางการเกษตร ซึ่งเป็นเชื้อที่ก่อโรคในสัตว์จำพวกม้า สุนัข และแมว แต่ช่วง 20 ปีที่ผ่านมา มีรายงานพบการติดเชื้อในมนุษย์ ส่วนใหญ่พบติดเชื้อในหลอดเลือดแดงบริเวณขา รอบดวงตา แต่มีอาการแตกต่างกัน อาทิ หากติดเชื้อบริเวณขา จะเกิดการอุดตันบริเวณเส้นเลือดแดง ทำให้เลือดไม่ไปเลี้ยงบริเวณขา หากรักษาไม่ทันจำเป็นต้องตัดขาทิ้ง ขณะที่การติดเชื้อบริเวณดวงตาจะเกิดแผลที่กระจกตา ลักษณะคล้ายต้อ หากรักษาไม่ทันแผลจะลุกลามและตาบอดได้

นพ.ธีรพงษ์ กระแจะจันทร์ ภาควิชาพยาธิวิทยา คณะแพทยศาสตร์ รพ.รามาธิบดี มหาวิทยาลัยมหิดล กล่าวว่า อุบัติการณ์การโรคพบไม่มากนัก ที่ผ่านมา รพ.รามาฯ ร่วมกับหน่วยงานภาคี อาทิ รพ.ศิริราช รพ.จุฬาลงกรณ์ ฯลฯ สำรวจผู้ป่วยที่ติดเชื้อพบเพียง 102 คน ในรอบ 20 ปี ส่วนใหญ่เป็นชาวนาที่ต้องทำงานในพื้นที่ชุ่มน้ำ ทำให้ติดเชื้อง่ายกว่าคนทั่วไป โดยพบมากที่สุดในภาคกลาง รองลงมาคือ ภาคอีสาน ภาคเหนือ ภาคตะวันออก และภาคใต้ตามลำดับ นอกจากนี้ จากการศึกษาที่ผ่านมายังพบว่า ผู้ป่วยโรคธาลัสซีเมียมีภาวะเสี่ยงต่อการติดเชื้อดังกล่าวมากกว่าคนปกติด้วย ซึ่งยังไม่ทราบสาเหตุที่แน่ชัด

ล่าสุด รพ.รามาธิบดี ร่วมกับสถาบันวิจัยจุฬาภรณ์ได้พัฒนาชุดตรวจวินิจฉัยโรคฮิวแมน พิธิโอซิส ขึ้น โดยพัฒนาโปรตีนจำเพาะที่สามารถตรวจจับเชื้อดังกล่าวได้ และทำการเคลือบโปรตีนชนิดนี้บนชุดตรวจที่มีลักษณะเหมือนชุดตรวจการตั้งครรภ์ เมื่อเจาะเลือดผู้ป่วย หากพบการติดเชื้อจะขึ้นแถบสีสองแถบ แต่หากไม่พบการติดเชื้อจะขึ้นเพียงแถบเดียว ใช้เวลาเพียง 30 นาทีเท่านั้น เบื้องต้นอยู่ระหว่างยื่นจดสิทธิบัตร

หน้า 5

วิทยาศาสตร์การแพทย์



ไทยรายแรกพัฒนาชุดตรวจ'พิธีโอซิส'

14 มกราคม พ.ศ. 2551 12:44:00

"พิธีโอซิส"โรคในกลุ่มเชื้อราที่ค้นพบใหม่ อาศัยในพื้นที่ชื้นแฉะที่มีพืชขึ้น ชาวนาชาวสวนจึงเป็นกลุ่มเสี่ยงสำคัญ และถ้ารักษาช้าอาจต้องตาบอดหรือตัดอวัยวะทิ้ง

กรุงเทพธุรกิจออนไลน์ : **น.พ. ธีรพงษ์ กระแจะจันทร์** คณะแพทยศาสตร์ โรงพยาบาลรามาธิบดี กล่าวว่า โรงพยาบาลรามาธิบดีร่วมกับ **ศ.ดร. กวี รัตนบรรณางกูร** จากสถาบันวิจัยจุฬาภรณ์ พัฒนาชุดตรวจ**โรคพิธีโอซิส** (Pythiosis) ในคนเป็นแห่งแรก

เนื่องจากเชื้อนี้เพิ่งค้นพบในต่างประเทศจึงมีข้อมูลไม่มาก ขณะที่ตัวเชื้อมีความรุนแรงสูง ผู้ป่วยอาจสูญเสียตาหรืออวัยวะที่ติดเชื้อได้ง่ายหากได้รับการรักษาล่าช้า

โรคฮิวแมนพิธีโอซิส เกิดจากการติดเชื้อ "**พิเธียม อินซิดิโอซุม**" (Pythium insidiosum) มีลักษณะเหมือนเชื้อรา เจริญเติบโตในพื้นที่ชื้นแฉะที่มีพืชอยู่ ที่ผ่านมาตรวจพบเชื้อนี้ในสัตว์เขตร้อน และเมื่อปี 2528 มีการรายงานโรคนี้ครั้งแรกจากโรงพยาบาลศิริราช จนถึงปัจจุบันพบผู้ป่วยประมาณ 102 คนจากโรงเรียนแพทย์ต่างๆ ส่วนใหญ่เป็นชาวนาเนื่องจากทำงานในน้ำหรือพื้นที่ชื้นแฉะ

"ที่ผ่านมาการวินิจฉัยโรคนี้ ต้องอาศัยการเพาะเนื้อเยื่อหาเชื้อโรค จึงทำได้เฉพาะในโรงพยาบาลใหญ่ รวมถึงใช้เวลาหลายวันกว่าจะทราบผล แต่**ชุดตรวจที่พัฒนาขึ้นนี้ใช้งานง่าย และที่สำคัญทราบผลได้ใน** 30 **นาทีเท่านั้น** จึงใช้ประโยชน์ได้ในสถานีอนามัยต่างจังหวัด แทนการส่งตัวอย่างเลือดมายังโรงพยาบาลใหญ่" น.พ.ธีรพงษ์ กล่าว

ทีมวิจัยอยู่ระหว่างยื่นจดสิทธิบัตร และมีแผนงานที่จะต่อยอดโดยดิดต่อผู้เชี่ยวชาญโรคนี้ในสัตว์จากมหาวิทยาลัยรัฐมิชิแกนสหรัฐ **เพื่อปรับปรุงให้สามารถใช้ตรวจโรคในสัตว์ เนื่องจากทั่วโลกยังไม่มีชุดตรวจโรคนี้**

ชุดตรวจโรคฮิวแมน พิธีโอซิส มีลักษณะเป็นแถบเหมือนกับชุดตรวจการตั้งครรภ์ ใช้งานโดยใส่ตัวอย่างโปรตีนที่สกัดได้จากเชื้อพิเธียมอินซิดิโอซุมลงในแถบวัด หากเลือดมีเชื้อดังกล่าวก็จะจับกับโปรตีนในแถบวัด และแสดงผลเป็นแถบคู่สีม่วง แต่หากเลือดไม่มีเชื้อ ก็จะปรากฏเป็นแถบสีม่วงเพียงแถบเดียว

ทีมวิจัยได้ทดสอบประสิทธิภาพของชุดตรวจ ในผู้ป่วยโรคฮิวแมนพิธีโอซิส 31 คน พบสามารถตรวจหาเชื้อโรคร้ายในเลือดของผู้ป่วย ที่แสดงอาการที่เส้นเลือด ผิวหนังและลำไส้ได้

แต่ไม่สามารถตรวจพบในผู้ป่วยที่แสดงอาการที่ตา เนื่องจากเลือดจากผู้ป่วยติดเชื้อในตา โดยทั่วไปจะไม่ทำปฏิกิริยากับชุดตรวจเลือด

ชุดตรวจนี้เป็นแนวทางหนึ่ง ที่ช่วยให้ผู้ป่วยได้รับการรักษาโดยเร็วและเพิ่มโอกาสหาย เนื่องจากปัจจุบันการรักษาหลักคือการผ่าตัดเอาเชื้อร้ายออกให้หมด เพื่อป้องกันเชื้อลุกลาม

เช่นเดียวกับการติดเชื้อในตาก็ต้องผ่าตัดเปลี่ยนกระจกตา จากนั้นผู้ป่วยก็จะกลับมามองเห็นได้เหมือนเดิม ส่วนขาก็อาจต้องผ่าตัดเส้นเลือดที่ติดเชื้อออก หากปล่อยไว้ให้อุดตันในเส้นเลือด เลือดไม่ไปเลี้ยงขาจนเนื้อเยื่อตาย ก็ต้องตัดขาทิ้งเพื่อไม่ให้ลุกลามต่อไป

อย่างไรก็ตามอาการเบื้องต้นที่สังเกตพบคือ เคืองตา ตาแดงมีหนอง หรือปวดขาเป็นระยะๆ หรือปวดเมื่อออกแรง กลุ่มเสี่ยงสำคัญคือผู้ที่สัมผัสพื้นที่ชื้นแฉะ เช่น ชาวนา รวมถึงผู้ป่วยธาลัสซีเมีย หากมีอาการดังกล่าวควรรีบพบแพทย์เพื่อตรวจวินิจฉัย

สาลินีย์ ทับพิลา

ข่าวที่เกี่ยวข้อง

- รับแก้ปัญหาภายใน สยบคลื่นนอกประเทศ
- สองเรื่องขบขันคลายเครียด...สิงคโปร์โต้ไทยแรง.. ห้ามเผ่าหัวงมาไทย?
- "ตั้ง "ชิโก้" ที่ปรึกษา รมต.ไทยได้ข่าวดีดิ้ม-โก่ฟิด"
- ลีลามือหนึ่ง
- แบดไทยเจองานหนักดบแดนโสม
- หอการค้าฯหนุนแก้ก.ม.ต่างด้าว ระบุกระทบธุรกิจต่างชาติ 15%
- 'พล.อ.สุรยุทธ์'ออกรายการช่อง11วันนี้เวลา11.00น.
- ดึงดาวดังปันส่วนเอเชียนทัวร์บุมแฟนเจ้าถิ่น
- มาตรการรองรับไทยเทียม
- "ทำไมคนไทยจึงล้าหลัง?" (1)"
- สิงคโปร์ : ทุนร้ายในอาเซียน
- จุฬาฯ แจงข้อเท็จจริงการเป็นมหาวิทยาลัยในกำกับของรัฐ
- กกท.เปิดรับเยาวชนร่วมลับฝีมือมุ่งสู่อาชีพ 5 มี.ค.ที่วอเตอร์มิลล์
- สวิงวันเดย์เปิดโอเพ่นชิงเงิน8แสนฤกษ์ดี 31 ม.ค.ที่เดอะวินเทจ
- มาเวอริคส์ชนะศึกเอ็นบีเอ
- ดั๊กแดนเสียงใสในละคร
- บทบันทึกริมฝั่งน้ำ
- สุดแดนเบื้องบูรพา 'สระแก้ว'
- Babel แห่ง Morocco
- 'ดิโม มูลเลอร์' สองล้อข้ามโลก
- ปฏิทินกิจกรรม ศุกร์ ที่ 19 มกราคม พ.ศ. 2550
- ไอดิน กลิ่นไวน์ ไร้องุ่นในฮันเตอร์วัลเลย์
- บ้านแบ่งเช่าในซอยอัครนิธิ 2
- เหงา
- หุหาเรื่อง
- ทฤษฎีการแพร่ข่าวลือ
- "สุวิทย์"เปิดช่องลดเวลาถือครอง-ขึ้นราคาบัตรอีลิท"
- กูรูชี้เอเชียรุกพัฒนาความร้องค์กรเทียบชั้นยุโรป
- หนุนดอบโต้
- 'ครม.-ปชช.'ร่วมทำบุญตักบาตรถวายเป็นพระราชกุศลแด่ในหลวง

เงื่อนไขในการแสดงความคิดเห็น

1. ทุกคนมีสิทธิ์ แสดงความคิดเห็นได้อย่างอิสระเสรี โดยไม่ใช้ ถ้อยคำหยาบคาย กล่าวพาดพิง
2. ทุกคนมีสิทธิ์ แสดงความคิดเห็นได้โดยความยาวไม่เกิน 500 ตัวอักษร ต่อ ความคิดเห็น
3. การลบความคิดเห็น ที่ไม่เหมาะสม สามารถกระทำได้ทันที โดยไม่ต้องมีการแจ้งให้ทราบล่วงหน้า
4. ความคิดเห็นเหล่านี้ ไม่สามารถนำไปอ้างอิงทางกฎหมาย และไม่เกี่ยวข้องใดๆทั้งสิ้นกับคณะผู้จัดทำเว็บไซต์

ร่วมแสดงความคิดเห็น

ชื่อ :

อีเมล์ :

ความคิดเห็น :

ตกลง ยกเลิก

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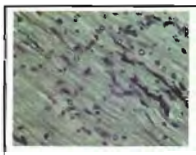
เก็บมาฝาก..จากบริหารงานวิจัย

การพัฒนาชุดตรวจวินิจฉัยโรค Human Pythiosis

Krajaejun, T., S. Imkhieo, A. Intaramat, K. Ratanabanangkoon. 2009. Development of an Immunochromatographic Test for Rapid Serodiagnosis of Human Pythiosis. Clin Vaccine Immunol. 16: 506-9.

โรค pythiosis เป็นโรคติดเชื้ออุบัติใหม่ ที่มีความรุนแรงถึงขั้นพิการหรือเสียชีวิต พบโรคนี้ได้ทั้งในคนและสัตว์ เช่น สุนัข แมว ม้า และวัว ที่อาศัยอยู่ในประเทศเขตร้อน สาเหตุเกิดจากการติดเชื้อ Pythium insidiosum ในภาวะแวดล้อมที่เหมาะสม เชื้อ P. insidiosum สามารถสร้าง zoospore ซึ่งมี flagella ช่วยในการเคลื่อนไหวในน้ำ Zoospore เป็นส่วนสำคัญของกระบวนการติดเชื้อ เพราะเมื่อ zoospore ว่ายน้ำมาสัมผัสกับผิวหนังหรือเยื่อบุของคนหรือสัตว์ จะงอกเป็นสายราชอนไชเข้าสู่เนื้อเยื่อที่อยู่ลึกลงไป และก่อให้เกิดพยาธิสภาพ ถึงแม้เชื้อ P. insidiosum มีลักษณะทางกายภาพเป็นสายราเหมือนเชื้อราทั่วๆไป แต่เชื้อ P. insidiosum มีลักษณะเฉพาะทาง physiology และ genetics ที่ไม่ถูกจัดอยู่ในกลุ่มเชื้อรา



แสดงเชื้อ P. insidiosum ในเนื้อเยื่อจากผู้ป่วย

โรค pythiosis ในคนนั้น พบครั้งแรกในประเทศไทย เมื่อปี พ.ศ. 2528 หลังจากนั้น มีรายงานการพบผู้ป่วยเพิ่มขึ้นเรื่อยๆ ส่วนใหญ่เป็นผู้ป่วยจากประเทศไทย

จึงถือได้ว่าประเทศไทยเป็น endemic area ของโรค pythiosis ในคน ลักษณะทางคลินิกของโรค pythiosis สามารถแบ่งได้เป็น 4 กลุ่ม คือ:

(1) ติดเชื้อที่ผิวหนัง (cutaneous/subcutaneous form): พบ 5% ของผู้ป่วยทั้งหมด ผู้ป่วยมาด้วยแผลเรื้อรังฝี-หนอง หรือการอักเสบของชั้นผิวหนังและใต้ผิวหนังบริเวณใบหน้า แขน หรือขา ส่วนใหญ่สามารถรักษาได้ด้วย saturated solution potassium iodide.

(2) ติดเชื้อที่เส้นเลือดแดง (vascular form): พบ 59% ของผู้ป่วยทั้งหมด ผู้ป่วยมาด้วย claudication หรือ gangrene ที่ขา เพราะมีการติดเชื้อที่ผนังหลอดเลือดแดงจนเกิดการอุดตัน ส่วนใหญ่ (~80% ของผู้ป่วย) รักษาโดยการตัดขาข้างที่ติดเชื้อทิ้ง ในรายที่มีการติดเชื้อรุกลามมากก็อาจเสียชีวิตได้จากการแตกของหลอดเลือดแดงใหญ่ (~40%)

แสดงผู้ป่วย vascular pythiosis คนหนึ่ง ที่ต้องรักษาโดยการตัดขาออกทั้ง 2 ข้าง (รูปได้รับความอนุเคราะห์จาก ศ.พญ. บุญมี สถาปัตยวงศ์)

(3) ติดเชื้อที่ตา (ocular form): พบ 33% ของผู้ป่วยทั้งหมด ผู้ป่วยมักจะมาด้วยแผลที่กระจกตา ประมาณ 2-3 สัปดาห์ การติดเชื้อมักลุกลาม ต้องรักษาโดยการผ่าตัดนำลูกตาข้างที่ติดเชื้อออก (~80% ของผู้ป่วย)

(4) ติดเชื้อบริเวณอื่น (disseminated form): พบ 3% ของผู้ป่วยทั้งหมด ผู้ป่วยในกลุ่มนี้พบติดเชื้อที่เนื้อสมอง ที่โพรงจมูกและไซนัส หรือที่กระเพาะและลำไส้ผู้ป่วยทั้งหมดในกลุ่มนี้เสียชีวิต

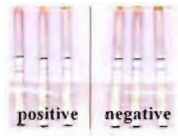
การกระจายของโรค human pythiosis ในประเทศไทย

จากการศึกษาทางระบาดวิทยา พบว่ามีการกระจายของโรคอยู่ทั่วประเทศ และมีปัจจัยบางประการที่สัมพันธ์กับผู้ป่วยโรค pythiosis เช่น การทำอาชีพเกษตรกรรม (~75% ของผู้ป่วย) ซึ่งเป็นอาชีพหลักของคนไทย, ช่วงอายุ 20-60 ปี (~86%)

ซึ่งเป็นช่วงวัยทำงาน, และเป็นเพศชาย (~71%). เป็นที่น่าสนใจว่าผู้ป่วยทุกคนที่มีรอยโรคที่เส้นเลือด ที่ผิวหนัง และที่อื่นๆ ยกเว้นที่ตา พบมีโรคประจำตัวเป็นโรคเลือด ที่พบมากที่สุด คือ thalassemia (~85%) ซึ่งเป็นโรคทางพันธุกรรมที่พบบ่อยในประเทศไทย ส่วนผู้ป่วยที่มีรอยโรคที่ตานั้น พบว่าส่วนใหญ่ (84%) ไม่มีโรคประจำตัว ข้อมูลนี้บ่งชี้ว่ามีปัจจัยบางประการที่ส่งเสริมให้ผู้ป่วยโรคเลือดติดเชื้อ P. insidiosum จนเกิดพยาธิสภาพได้ แต่อย่างไรก็ตาม ผู้ที่มีสุขภาพแข็งแรง ไม่มีโรคประจำตัว ก็สามารถติดเชื้อ P. insidiosum ได้เช่นกัน

ปัญหาสำคัญของโรค pythiosis คือ มีอัตราทุพพลภาพและอัตราการตายที่สูง ซึ่งส่วนหนึ่งเกิดจากขาด diagnostic test ที่มีประสิทธิภาพ และง่ายต่อการใช้ เพื่อใช้ช่วยวินิจฉัยโรคที่ถูกต้องและรวดเร็ว อันจะทำให้การรักษาสามารถทำได้รวดเร็วขึ้นด้วย ในปัจจุบัน วิธีวินิจฉัยที่นิยม คือ การเพาะเชื้อ หรือการตรวจเลือดหา antibody ต่อเชื้อ P. insidiosum ด้วยวิธีการ immunodiffusion test ซึ่งเป็นวิธีการที่ต้องใช้เวลา อาศัยผู้มีความชำนาญ และมีความไวต่ำ จึงทำให้มีโอกาสเกิด false negative ได้มาก จากปัญหาการวินิจฉัยดังกล่าว จึงได้มีการพัฒนาชุดทดสอบที่มีประสิทธิภาพมากขึ้นที่เรียกว่า immunochromatography test (ICT) เพื่อตรวจหา antibody ต่อเชื้อ P. insidiosum จากเลือดผู้ป่วย ชุดทดสอบนี้ใช้หลักการเดียวกับชุด strip ทดสอบการตรวจการตั้งครรภ์จากปัสสาวะที่มีใช้กันทั่วไป

ในการผลิตชุดทดสอบ ICT สำหรับวินิจฉัยโรค pythiosis ต้องใช้ antigen ที่สกัดได้จากเชื้อ P. insidiosum ในห้องทดลอง และใช้ colloidal gold เป็นตัวให้สัญญาณ การแปลผลนั้นทำได้ง่าย คือ เมื่อทดสอบเลือดผู้ป่วย pythiosis จะเกิดเป็นแถบสีม่วง 2 แถบ ในขณะที่เมื่อทดสอบเลือดของผู้คนที่ไม่ได้ติดเชื้อจะเกิดแถบสีม่วงเพียงแถบเดียว เมื่อทดสอบประสิทธิภาพของ ICT เปรียบเทียบกับ immunodiffusion test (ชุดทดสอบที่นิยมใช้สำหรับโรค pythiosis) โดยใช้เลือดจากผู้ป่วย pythiosis จำนวน 33 ราย (แบ่งเป็น vascular pythiosis 27 ราย, ocular pythiosis 4 ราย และ cutaneous pythiosis 2 ราย) และเลือดจากกลุ่ม control จำนวน 181 ราย (แบ่งเป็นเลือดจากผู้มาบริจาคเลือดที่มีร่างกายแข็งแรง 100 ราย, ผู้ป่วยติดเชื้อชนิดอื่นๆ 56 ราย, thalassemia 19 ราย, และผู้ป่วยโรค autoimmune diseases 6 ราย) เมื่อตรวจเลือดจากกลุ่ม control พบว่าทั้งหมดให้ผลลบ เมื่อทดสอบด้วย ICT หรือ immunodiffusion test แต่เมื่อตรวจเลือดจากกลุ่มผู้ป่วย pythiosis พบว่า immunodiffusion test ให้ผลลบกับผู้ป่วย vascular pythiosis 8 ราย และ cutaneous pythiosis 1 ราย ในขณะที่ ICT ให้ผลบวกในผู้ป่วยเหล่านี้ เป็นที่น่าสังเกตว่า ทั้ง ICT และ immunodiffusion test ให้ผลลบกับผู้ป่วย ocular pythiosis ทุกราย ซึ่งสอดคล้องกับการติดเชื้อที่ตามักกระตุ้นการสร้าง antibody ได้น้อย และการตรวจหาระดับ antibody จึงไม่เหมาะกับการวินิจฉัยการติดเชื้อที่ตา. เมื่อดูระยะเวลาที่ใช้ในการทดสอบ พบว่า ICT ใช้เวลาในการทดสอบสั้นกว่า immunodiffusion test อย่างมาก (30 นาที vs. 24 hours). โดยสรุปแล้ว, ICT และ immunodiffusion test เป็น tests ที่มี specificity ดีมาก (100% ทั้ง 2 tests) แต่ ICT มี sensitivity ที่ดีกว่า immunodiffusion test อย่างชัดเจน (88% สำหรับ ICT และ 61% สำหรับ immunodiffusion test). ดังนั้น ICT จึงเป็นชุดตรวจวินิจฉัยโรค vascular และ cutaneous pythiosis ที่มีประสิทธิภาพกว่า immunodiffusion test ทั้งยังสามารถอ่านผลได้รวดเร็ว และง่ายต่อการใช้โดยผู้ใช้ไม่จำเป็นต้องมีประสบการณ์ทางห้องปฏิบัติการมากนัก จึงเหมาะที่จะนำ ICT ไปใช้ช่วยวินิจฉัยโรค pythiosis และสามารถนำไปใช้ในพื้นที่ห่างไกลที่มีโอกาสพบผู้ป่วยได้มาก



รูปแสดง immuno-chromatographic test

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