รายงานวิจัยฉบับสมบูรณ์

โครงการ: การพิสูจน์หาหน้าที่ของ Putative Exo-1,3-β Glucanase Gene ของเชื้อ *Pythium insidiosum*

(Functional Characterization of the Putative Exo-1,3-β Glucanase Gene of the Human Pathogenic Oomycete *Pythium insidiosum*)

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มหาวิทยาลัยมหิดล

เดือน ปี ที่เสร็จโครงการ: มิถุนายน 2556

สัญญาเลขที่ RMU5380047

รายงานวิจัยฉบับสมบูรณ์

โครงการ

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สนับสนุนโดยสำนักงานคณะกรรมการการอุดมศึกษา
และสำนักงานกองทุนสนับสนุนการวิจัย

สารบัญ

ABSTRACT

Pythium insidiosum is the etiologic agent of pythiosis, an emerging and fatal infectious disease of humans and animals living in tropic and subtropic countries. *P. insidiosum* exists in two stages: branching hyphae and biflagellate zoospore. Although morphology of *P. insidiosum* resembles to fungi, but it is closely related to diatom and algae. Human pythiosis is found mostly in Thailand, while the disease state in animals is found worldwide. Thalassemia is a predisposing factor. Healthcare professionals are unfamiliar with the disease. Diagnosis of pythiosis is difficult. Conventional antifungal drugs are ineffective, leaving radical surgery of infected organ is the main treatment option for pythiosis. 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. Recently, we have identified a 74-kDa immunoreactive protein of *P. insidiosum*. Proteomic and genetic analyses revealed a partial *P. insidiosum* exo-1,3-ß-glucanase gene (*PinsEXO1*) sequence that encodes this immunogen. In pathogenic fungi, glucanases have roles in morphogenesis, pathogenesis, and host immune responses. Role of the gucanase in *P. insidiosum* is unknown. *PinsEXO1* might have functions resembled to fungal glucanases, and could be a potential virulence factor, diagnostic target, and vaccine candidate. In this project, we aims to characterize *PinsEXO1* and apply the gene for clinical uses. Full-length *PinsEXO1* (2,229 bp) was successfully identified, using Adaptor and RACE PCR. Sequence analysis showed that *PinsEXO1* was phylogenetically different from fungal glucanases. *PinsEXO1* was significantly up-regulated when it grew at body temperature, compared to room temperature. Limited carbon source down-regulated *PinsEXO1.* We showed that *PinsEXO1* was an efficient DNA target for specific PCR identification of *P. insidiosum*. Two B-cell epitopes of PinsEXO1 protein (Peptide-A and B), predicted by bioinformatics analysis, were strongly reactive against sera from pythiosis patients, but not control sera tested. Western blot analysis of hyphal and culture filtrate antigens, using rabbit anti-Peptide-A/B serum, showed that PinsEXO1 was not secreted. Immunostaining suggested that PinsEXO1 localized at cell wall. By comparing the two peptides, Peptide-A was a more suitable epitope for development of an efficient peptide-based ELISA for diagnosis of pythiosis. In conclusion, the glucanase gene, *PinsEXO1*, of *P. insidiosum*, was first immunologically and genetically characterized here. Highly-expressed *PinsEXO1* at body temperature and surface localization of its protein imply that the gene might have role in host interaction. *PinsEXO1* was proven to be useful for molecular and immunological detection of *P. insidiosum*.

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*. เมื่อไม่นานมานี้ คณะผู้วิจัยได้รายงานการพบ immunodominant protein ขนาด 74-kDalton ของเชื้อ *P. insidiosum* และจากการวิเคราะห์ทางด้าน Proteomic and genetic พบยีนที่ถอดรหัสเป็นโปรตีนนี้คือ exo-1,3-β-glucanase gene (*PinsEXO1*). Glucanase เป็นโปรตีนที่น่าสนใจ เพราะในเชื้อราบางชนิด glucanase มีบทบาทในการเปลี่ยนแปลงรูปร่างของเชื้อ, ช่วยก่อให้เกิดโรค (การเกาะติดและการชอนไชเนื้อเยื่อโฮส), และมีบทบาทในการกระตุ้นภูมิกันของร่างกาย. หน้าที่ของ *P. insidiosum* glucanas อาจจะเหมือนกับที่พบในเชื้อรา แต่ก็ยังไม่มีการพิสูจน์. วัตถุประสงค์ของโครงการนี้คือ เพื่อศึกษาหน้าที่ของ *P. insidiosum PinsEXO1* และใช้ประโยชน์จากยีนนี้ในทางคลินิก. จากการใช้เทคนิค Adaptor and RACE PCR ทำให้สามารถทราบลำดับเบสของ *PinsEXO1* ทั้งหมด. เมื่อศึกษาความสัมพันธ์ทาง phylogenetics พบว่า *PinsEXO1* มีลำดับเบสแตกต่างกับเชื้อราชนิดอื่นๆ. เมื่อดูการแสดงออกของยีน พบว่า *PinsEXO1* แสดงออกสูงเมื่อเชื้อเจริญที่อุณหภูมิร่างกาย และมีการแสดงออกต่ำเมื่อเชื้อเจริญในภาวะที่ขาดคาร์บอน. คณะผู้วิจัยได้ใช้ *PinsEXO1* เป็นเป้าหมายในการตรวจหา *P. insidiosum* ด้วยวิธี PCR พบว่ามีประสิทธิภาพดี. นอกจากนี้ยังใช้วิธีทาง Bioinformatics คำนาย B-cell epitopes ได้ 2 epitopes คือ Peptide-A และ -B ซึ่งมี binding activity ดีกับ anti-*P. insidiosum* antibody ใน serum ผู้ป่วย pythiosis ทั้งหมดที่ทดสอบ. จากการทำ Western blot analysis กับ crude antigens ที่สกัดได้จากเชื้อ ร่วมกับ antibody จากกระต่ายที่สร้างต่อ Peptide-A และ –B พบว่าโปรตีน PinsEXO1 ไม่มีการหลั่งออกมานอกเซลล์ และการใช้ immunostaining assay บ่งบอกว่าโปรตีน PinsEXO1 อยู่ที่ผนังเซลล์. เมื่อเปรียบเทียบ Peptide-A และ –B เพื่อใช้ทำ Peptide ELISA สำหรับวินิจฉัยโรค Pythiosis พบว่า Peptide-A มีประสิทธิภาพดีกว่า. โดยสรุป โครงการวิจัยนี้ได้ทำการศึกษายีน *PinsEXO1* ของ *P. insidiosum* ในด้านที่เกี่ยวกับภูมิคุ้มกันวิทยาและยีเนติกส์เป็นครั้งแรก และแสดงว่ายีน *PinsEXO1* อาจมีบทบาทต่อการเกิดโรค. นอกจากนี้ยีน *PinsEXO1* ยังสามารถนำมาประยุกต์ใช้เพื่อการวินิจฉัยการติดเชื้อ *P. insidiosum* ได้อย่างดี.

เนื้อหางานวิจัย

1. รหัสโครงการ RMU5380047

2. ชื่อโครงการ

การพิสูจน์หาหน้าที่ของ Putative Exo-1,3-β Glucanase Gene ของเชื้อ *Pythium insidiosum* (Functional Characterization of the Putative Exo-1,3-β Glucanase Gene of the Human Pathogenic Oomycete *Pythium insidiosum*)

3. ชื่อหัวหน้าโครงการ

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งบประมาณทั้งโครงการ: 1,200,000 บาท

4. ระยะเวลาโครงการ: 3 ปี (15 มิถุนายน 2553 – 14 มิถุนายน 2556)

5. Keywords: *Pythium insidiosum*; Pythiosis; Glucanase; Characterization; Diagnostic assay

6. บทนำ

6.1 Background and significance of human pythiosis

โรค human pythiosis เป็นโรคติดเชื้ออุบัติใหม่ (emerging infectious disease) [1–4]. รายงานผู้ป่วยเกือบทั้งหมดมาจากประเทศไทย [4]. นอกจากพบในคนแล้ว ยังพบโรคนี้ในสัตว์ด้วย เช่น สุนัข แมว ม้า และวัว ที่อาศัยอยู่ในประเทศเขตร้อน [1–3]. โรค pythiosis มีความรุนแรงถึงขั้นพิการหรือเสียชีวิต เกิดจากการติดเชื้อ *Pythium insidiosum* ซึ่งเป็นเชื้อในกลุ่ม oomycetes ที่จัดอยู่ใน Phylum Pseudofungi, Kingdom Chromista (Stramenopila) [3]. เชื้อในกลุ่ม oomycetes มีลักษณะทาง physiology และ genetics เฉพาะตัว จึงถูกจัดแยกจาก microorganisms อื่นๆ เช่น fungi, viruses, bacteria หรือ parasites [5]. ในธรรมชาติ เชื้อ *P. insidiosum* อาศัยอยู่ในน้ำหรือที่ชื้นแฉะ ในรูปของสายรา (**Figure INTRO-1**) [6]. เมื่อมีสิ่งแวดล้อมที่เหมาะสม เชื้อ *P. insidiosum* สามารถสร้าง zoospore (**Figure INTRO-1**) ซึ่งมี flagella ช่วยในการว่ายน้ำ. Zoospore เป็นส่วนสำคัญของกระบวนการติดเชื้อ เพราะ zoospore สามารถว่ายน้ำไปเกาะผิวหนังหรือเยื่อบุต่างๆ ของคนหรือสัตว์ และงอกเป็นสายรา เพื่อชอนไชไปสู่เนื้อเยื่อที่อยู่ลึกลงไป และก่อให้เกิดพยาธิสภาพ [6].

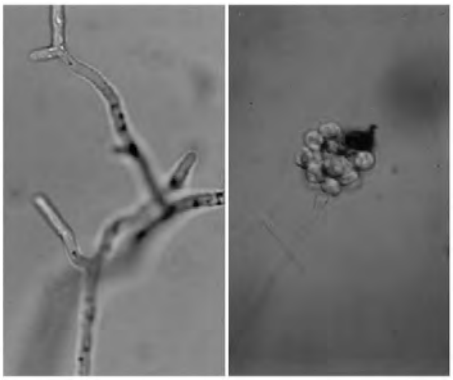


Figure INTRO-1. รูปถ่ายจากกล้อง light microscope แสดงลักษณะของเชื้อ *Pythium insidiosum*: (**A**) สายรา (hphae) และ (**B**) zoospores sac ภายในมี zoospores อยู่ [7].

รายงานการพบโรค human pythiosis ครั้งแรกมาจากประเทศไทยในปี พ.ศ. 2528 [2,4,8]. จากนั้นโรคนี้ก็เริ่มเป็นที่รู้จักและมีการรายงานผู้ป่วยเพิ่มขึ้นเรื่อยๆ ส่วนใหญ่เป็นผู้ป่วยจากประเทศไทย [2,4,7–16]. จึงถือได้ว่าประเทศไทยเป็น endemic area ของโรค pythiosis ในคน. เนื่องจาก human pythiosis เป็นโรคที่รู้จักได้ไม่นาน ข้อมูลเกี่ยวกับโรคนี้ยังน้อยมาก และบุคลากรทางการแพทย์ส่วนใหญ่อาจจะยังไม่คุ้นเคยกับโรค ดังนั้นข้าพเจ้าและคณะฯ จึงได้รวบรวมข้อมูลผู้ป่วยโรค pythiosis ที่วินิจฉัยระหว่างปี พ.ศ. 2528 ถึง 2546 จาก 9 โรงพยาบาลทั่วประเทศ มาวิเคราะห์ทางคลินิกและทางระบาดวิทยา [4] เพื่อให้เข้าใจโรคในทางคลินิกมากขึ้น และเพื่อเป็นข้อมูลพื้นฐานสำหรับการวิจัยแง่มุมอื่น. ในการศึกษานี้ พบผู้ป่วยจำนวน 102 ราย โดย 40% ของผู้ป่วย ได้รับการวินิจฉัยในช่วง 3-4 ปีสุดท้ายของการศึกษา แสดงถึงแนวโน้มที่จะพบโรค pythiosis มากขึ้นในอนาคต. ลักษณะทางคลินิกของโรค pythiosis สามารถแบ่งได้เป็น 4 กลุ่ม คือ:

(1) *ติดเชื้อที่ผิวหนัง (cutaneous/subcutaneous form)*: พบ 5% ของผู้ป่วยทั้งหมด. ผู้ป่วยมาด้วยแผลเรื้อรัง ฝี-หนอง หรือการอักเสบของชั้นผิวหนังและใต้ผิวหนังบริเวณใบหน้า แขน หรือขา. สามารถรักษาได้ด้วย saturated solution potassium iodide.

(2) *ติดเชื้อที่เส้นเลือดแดง (vascular form)*: พบ 59% ของผู้ป่วยทั้งหมด. ผู้ป่วยมาด้วย claudication หรือ gangrene ที่ขา เพราะมีการติดเชื้อที่ผนังหลอดเลือดแดงจนเกิดการโป่งพองหรืออุดตัน (**Figure INTRO-2**). ส่วนใหญ่ (~80% ของผู้ป่วย) รักษาโดยการตัดขาข้างที่ติดเชื้อออก. ในรายที่มีการติดเชื้อรุกลามมากก็อาจเสียชีวิตได้จากการแตกของหลอดเลือดแดงใหญ่ (~40% ของผู้ป่วย).

(3) *ติดเชื้อที่ตา (ocular form)*: พบ 33% ของผู้ป่วยทั้งหมด. ผู้ป่วยมักจะมาด้วยแผลที่กระจกตาประมาณ 2-3 สัปดาห์ (**Figure INTRO-3**). การติดเชื้อมักลุกลาม ต้องรักษาโดยการผ่าตัดนำลูกตาข้างที่ติดเชื้อออก (~80% ของผู้ป่วย).

(4) *ติดเชื้อบริเวณอื่น (miscellaneous form)*: พบ 3% ของผู้ป่วยทั้งหมด. ผู้ป่วยในกลุ่มนี้พบติดเชื้อที่เนื้อสมอง ที่โพรงจมูกและไซนัส หรือที่กระเพาะและลำไส้. ผู้ป่วยทั้งหมดในกลุ่มนี้เสียชีวิต.

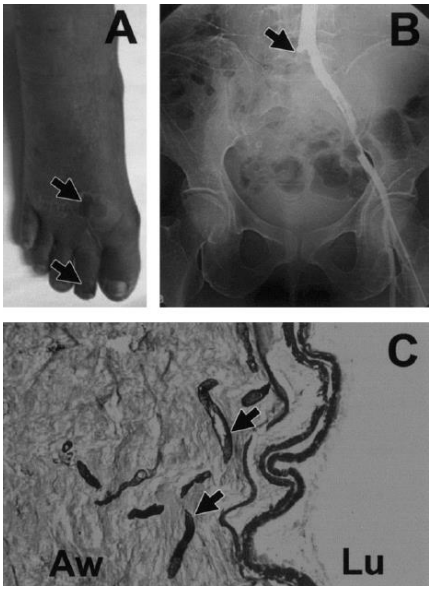


Figure INTRO-2. Typical clinical and pathological features of vascular pythiosis. ***A,*** Patient with thalassemia presenting with gangrenous ulcer *(arrows)* of lower extremities. ***B,*** Angiography showing occlusion *(arrow)* of a medium-to-large size artery. ***C,*** Histopathological examination of infected artery *(arrows)* by Gomori methenamine silver (GMS) staining. [Aw, arterial wall; Lu, arterial lumen] [4].

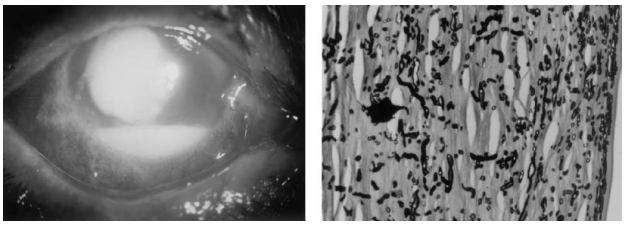


Figure INTRO-3. Clinical presentation and pathology of ocular pythiosis. **(Left)** An infected eye. **(Right)** Histological section showing hyphal elements appeared in full-thickness corneal stroma (GMS;x40) [7].

จากการศึกษาทางระบาดวิทยา พบมีผู้ป่วยกระจายอยู่ทั่วประเทศ (**Figure INTRO-4**) แสดงว่ามีการกระจายของเชื้อ *P. insidiosum* อยู่ทั่วประเทศ [4]. และจากลักษณะภูมิศาสตร์ จึงน่าจะมีการกระจายของเชื้อที่ประเทศเพื่อนบ้านด้วย (แต่ยังไม่มีการรายงานโรคจากประเทศดังกล่าว ยกเว้นประเทศมาเลเซีย ที่พบผู้ป่วยติดเชื้อที่ตา [17]). ยังพบด้วยว่า มีปัจจัยบางประการที่สัมพันธ์กับผู้ป่วยโรค pythiosis เช่น การทำอาชีพเกษตรกรรม (75% ของผู้ป่วย) ซึ่งเป็นอาชีพหลักของคนไทย, ช่วงอายุ 20-60 ปี (86%) ซึ่งเป็นช่วงวัยทำงาน, และเป็นเพศชาย (71%) [4]. เป็นที่น่าสนใจว่าผู้ป่วย cutaneous, vascular และ miscellaneous pythiosis ทุกคน มี underlying hematological disorders ส่วนใหญ่คือ thalassemia (85%) ซึ่งเป็นโรคทางพันธุกรรมที่พบบ่อยโรคหนึ่งในประเทศไทย [4,11]. ส่วนผู้ป่วย ocular pythiosis นั้น ส่วนใหญ่ (84%) ไม่พบมีโรคประจำตัวใดๆ [4,7]. ข้อมูลนี้จึงแนะว่ามีปัจจัยบางประการที่ส่งเสริมให้ผู้ป่วยโรคเลือด ติดเชื้อ *P. insidiosum* จนเกิดพยาธิสภาพ. อย่างไรก็ตาม ผู้ที่มีสุขภาพแข็งแรง ไม่มีโรคประจำตัว ก็สามารถติดเชื้อ *P. insidiosum* ได้.

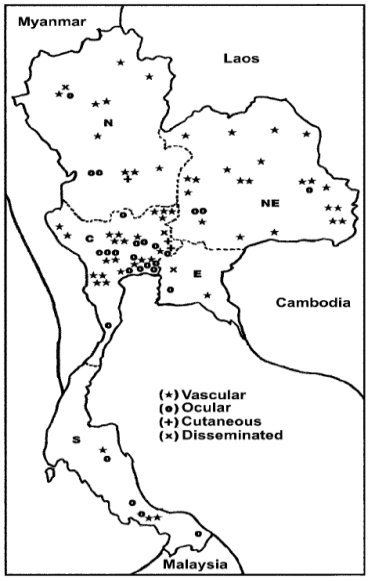


Figure INTRO-4. Geographic distribution of patients with pythiosis in Thailand. C, central region; E, eastern region; N, northern region; NE, northeastern region; S, southern region [4].

ปัญหาสำคัญของโรค pythiosis คือ การมีอัตราทุพพลภาพและอัตราการตายที่สูงเนื่องจาก (i) บุคลากรทางการแพทย์ไม่รู้จักโรคทำให้วินิจฉัยล่าช้า, (ii) ขาด diagnostic test ที่มีประสิทธิภาพ ให้ผลรวดเร็วและง่ายต่อการใช้, (iii) การรักษาทางยาที่มีอยู่ในปัจจุบันใช้ไม่ได้ผล และ (iv) วัคซีนที่ใช้รักษาโรคมีประสิทธิภาพต่ำ [4,12,18]. การรักษาหลักในปัจจุบัน คือ การผ่าตัดเนื้อเยื่อหรืออวัยวะที่ติดเชื้อออกให้หมด ซึ่งทำให้เกิดความพิการ [4]. ผู้ป่วยหลายรายเสียชีวิตถึงแม้จะรักษาอย่างดีที่สุดแล้ว. ดังนั้น จึงจำเป็นต้องทำวิจัยทางด้าน basic research เพื่อให้เข้าใจชีววิทยา และพยาธิกำเนิดของโรคอย่างลึกซึ้ง. เพราะ basic research เป็นแนวทางที่จะได้องค์ความรู้ใหม่เกี่ยวกับโรคนี้ ที่จะสามารถนำไปประยุกต์ใช้ดูแลรักษาผู้ป่วยที่ดีขึ้น เช่น มีวิธีป้องกันการเกิดโรค, มีวิธีวินิจฉัยที่ถูกต้องรวดเร็ว, ทราบ drug target ที่จะพัฒนายาใหม่เพื่อใช้รักษาโรค และพบ candidate สำหรับพัฒนา vaccine เป็นต้น. งานวิจัยของข้าพเจ้าขณะนี้จึงเน้นการศึกษา biology และ pathogenesis ของ *P. insidiosum* โดยใช้เทคนิคทางด้าน cellular/molecular และ bioinformatics. ผลงานวิจัยเบื้องต้น และ preliminary data ที่ผ่านมามีดังนี้:

6.2 Identification of a 74-kDa immunodominant antigen of *P. insidiosum* as a key candidate for clinical applications and biological study

ข้าพเจ้าและคณะฯ ได้วิเคราะห์หาโปรตีนของเชื้อ *P. insidiosum* ที่มีความสำคัญทางภูมิคุ้มกันวิทยา (immunodominant antigen) ด้วย Western blot analysis และได้พบโปรตีน immunodominant antigen ขนาด 74 kDa ในเชื้อ *P. insidiosum* ทั้ง 16 isolates ที่แยกได้จากผู้ป่วยจากทั่วประเทศ (**Figure**

INTRO-5) [19]. โปรตีน 74 kDa นี้ เป็นเพียงโปรตีนเดียวที่ตรวจพบได้ด้วย sera จากผู้ป่วยโรค pythiosis ทุกคนที่ใช้ทดสอบ โดยไม่พบมี cross reaction กับ control sera จากผู้ป่วยติดเชื้อชนิดอื่น. โปรตีน 74kDa จึงเป็น common immunodominant antigen ของเชื้อ *P. insidiosum* ที่มีความจำเพาะและเหมาะสำหรับพัฒนาทำ diagnostic test. นอกจากนี้ จากการวิเคราะห์เปรียบเทียบด้วย Western blot ไม่พบโปรตีน 74kDa นี้ใน *Pythium deliense* และ *Pythium aphanidermatum* ซึ่งเป็น *Pythium* species ที่มีความสัมพันธ์ทาง phylogenetics ใกล้ชิดกับ *P. insidiosum* แต่*ไม่*ก่อโรคในคนหรือสัตว์. ดังนั้น โปรตีน 74kDa จึงอาจมีบทบาทสำคัญทาง biology และ/หรือ pathogenesis ของเชื้อ.

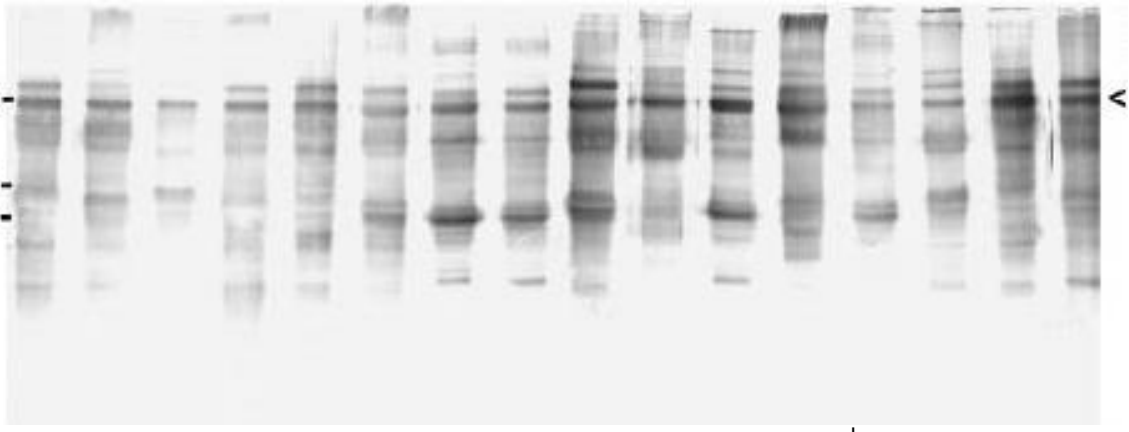


Figure INTRO-5. แสดง Western blot analysis ของโปรตีนที่สกัดจาก *P. insidiosum* จำนวน 16 isolates ที่แยกด้วยวิธี SDS-PAGE และทำปฏิกิริยากับ serum ของผู้ป่วย pythiosis. ลูกศรชี้ immunodominat protein ขนาด 74 kDa ซึ่งสามารถตรวจพบได้ในทุกสายพันธุ์ที่ทดสอบ [19].

6.3 The 74-kDa immunoreactive antigen is a putative exo-1,3-beta glucanase

คำถามวิจัยต่อไป คือ immunodominant antigen ขนาด 74 kDa คือโปรตีนอะไร? และยีนที่ encode โปรตีน 74 kDa นี้มี sequence อย่างไร? เพื่อตอบคำถามนี้ ข้าพเจ้าใช้วิธีทาง proteomics [20,21] เพื่อวิเคราะห์หาชนิดโปรตีน 74kDa. ขั้นแรกแยก crude protein extract ของเชื้อ *P. insidiosum* ด้วย SDS-PAGE, ตัดแยกโปรตีนขนาด 74 kDa, และย่อยเป็น peptides สายสั้นๆ ด้วยเอนไซม์ trypsin เพื่อใช้วิเคราะห์หาลำดับ amino acid ของสาย peptides ด้วย mass spectrometric analysis (MALDI-TOF/TOF). พบมี 2 peptides ที่มี amino acid sequence ตรงกับ exo-1,3-beta glucanase ของเชื้อ *Phytophthora infestans* [22] (*P. infestans* เป็นเชื้ออยู่ในกลุ่ม oomycetes เช่นเดียวกับ *P. insidiosum* แต่ก่อโรคในพืชเท่านั้น). จากนั้นจึงใช้ amino acid sequences ทั้งสองนี้เพื่อออกแบบ degenerate primers เพื่อทำ PCR amplification ส่วนหนึ่งของยีนที่ encode โปรตีน 74 kDa นี้จาก genomic DNA ของเชื้อ *P. insidiosum*. PCR product ที่ได้เป็น partial gene sequence มีขนาด 1.1 kb และมี

sequence คล้ายกับยีน exo-1,3-beta glucanase ของเชื้อ *P. infestans* ด้วยเช่นกัน (76% identity and 86% similarity).

Glucanases เป็นกลุ่มของ enzymes ที่พบในสิ่งมีชีวิตหลายชนิด เช่น รา [23–25]. Fungal glucanase ช่วยย่อย glucan ซึ่งเป็นส่วนประกอบหลักที่แข็งแรงของ cell wall เพื่อให้เกิด cell wall remodeling และราสามารถงอกเป็นสายราเพื่อ growth ได้ [23–25]. ใน *Candida albicans* พบว่า glucanase เป็น immunoreactive antigen อยู่ที่ใน cell wall และทำหน้าที่เป็น adhesive molecular มีส่วนในการยึดติดกับ host [26]. พบว่า *C. albicans* glucanase มีส่วนสำคัญในการกระตุ้น host immune responses [27–29]. เมื่อ glucanase gene ถูก disrupted ไป *Candida* จะไม่สามารถยึดเกาะกับ host ได้, มี hyphal growth defect, และเสียความสามารถในการก่อโรคในสัตว์ทดลองไป [26]. ดังนั้น glucanase ในเชื้อราจึงมีบทบาทต่อ host immune response, morphogenesis และ pathogenesis. ใน oomycetes มีการรายงานการพบ putative glucanase genes ใน *P. infestans* [22] แต่บทบาทหน้าที่ของ glucanase genes ใน oomycetes ยังไม่มีการศึกษา.

6.4 Roles of the putative exo-1,3-beta glucanase gene of *P. insidiosum*

เนื่องจาก glucanase มีหน้าที่ทางด้าน biology และ pathogenesis หลายประการในเชื้อรา แต่หน้าที่ของ enzyme นี้ใน *P. insidiosum* ร่วมทั้ง oomycetes ชนิดอื่น ยังไม่มีข้อมูล. ดังนั้น วัตถุประสงค์สำหรับโครงการวิจัยนี้คือเพื่อตอบคำถามเกี่ยวกับหน้าที่และการประยุกต์ใช้ glucanase gene ดังนี้ glucanase ของ *P. insidisoum* มี coding sequence และ functional domains (gene anatomy) อย่างไร, มีลำดับเบสสัมพันธ์กับ glucanase gene ของเชื้อ oomycetes และ fungi อื่น ในเชิง phylogenetics อย่างไร, มีการแสดงออก (gene transcription) ที่แตกต่างกันใน growth condition ต่างๆ หรือไม่ อย่างไร, มีตำแหน่งอยู่ที่ไหนของเซล (cellular localization), มีคุณสมบัติในการย่อยสลาย beta-glucan หรือไม่, มีบทบาทต่อการงอกของสายราหรือไม่, เป็น diagnostic target ที่จำเพาะเหมาะต่อการนำไปผลิตชุดวินิจฉัยหรือไม่มี functions อะไร?

โดยสรุป: ถึงแม้ *P. insidiosum* มีลักษณะเป็นสายรา แต่ก็ไม่จัดอยู่ในกลุ่มรา แต่ถูกจัดอยู่ในกลุ่ม oomycetes ที่มีลักษณะเฉพาะ เช่น สร้าง biflagellate zoospore ซึ่งสามารถว่ายน้ำได้. เชื้อในกลุ่ม oomycetes ส่วนใหญ่จะก่อโรคในพืช ส่วนน้อยก่อโรคในสัตว์ และมีเพียง species เดียว คือ *P. insidiosum* ที่ก่อโรคในคน. การก่อโรค ของเชื้อกลุ่ม oomycetes เป็นปัญหาใหญ่กับการเกษตร รวมถึงการแพทย์ เพราะก่อเกิดความสูญเสียพืชหรือสัตว์เศรษฐกิจจำนวนมาก และก่อให้เกิดโรคที่มีความรุ่นแรงที่ถึงขั้นพิการหรือเสียชีวิตในคนและสัตว์ โดยที่ยังไม่มีวิธีควบคุม-รักษาที่มีประสิทธิภาพ. ข้อมูลวิจัยในเชื้อกลุ่ม oomycetes ยังมีน้อยมากเมื่อเทียบกับกลุ่ม fungi. ดังนั้น การศึกษาเชื้อ oomycetes เพื่อให้เข้าใจถึงกลไกการก่อโรค เพื่อใช้ควบคุมโรคได้จึงจำเป็น.

งานวิจัยของข้าพเจ้าเน้นศึกษา *P. insidiosum* และใช้ human pathogen นี้เป็น model ในการศึกษา oomycete biology. การศึกษา glucanase gene จะเป็นจุดเริ่มต้นเรียนรู้ biology และ pathologenesis ระดับโมเลกุลในเชื้อ *P. insidiosum* เพื่อให้ได้องค์ความรู้ใหม่ที่ใช้เชื่อมโยงหรือประยุกต์กับความรู้เดิมเกี่ยวกับ gene/enzyme นี้ใน pathogenic oomycetes หรือ fungi อื่น. การศึกษา glucanase gene ของ *P. insidiosum* สามารถนำมาประยุกต์ใช้ทางคลินิกได้ เช่น การศึกษาทาง molecular epidemiology หรือ phylogeny, การพัฒนาชุดตรวจวินิจฉัย, การหา target ของยาที่เหมาะสมที่ครอบคลุมเชื้อราได้หลายชนิด, และการพัฒนาวัคซีนเพื่อใช้รักษาโรค. นอกจากนี้ ผลของการศึกษาจะสามารถใช้ตีพิมพ์ในวารสารวิชาการระดับนานาชาติที่มี impact ได้.

7. วัตถุประสงค์ของโครงการ

- เพื่อวิเคราะห์หา full length sequence ของ glucanase gene ของ *P. insidiosum*, และเปรียบเทียบความแตกต่างกับเชื้อราชนิดอื่นๆ
- เพื่อหา cellular localization ของ glucanase ในเชื้อ *P. insidiosum*
- เพื่อศึกษา glucanase gene expression ของเชื้อ *P. insidiosum* ในสภาวะต่างกัน
- เพื่อนำ putative glucanase ของเชื้อ *P. insidiosum* มาพัฒนาเป็น diagnostic assays
- เพื่อศึกษาว่า putative glucanase ของเชื้อ *P. insidiosum* มี glucanase activity หรือไม่
- เพื่อพัฒนา molecular genetic tools สำหรับใช้ศึกษา functional genetics ของเชื้อ *P. insidiosum*
- เพื่อศึกษาว่า putative glucanase มีบทบาทต่อการงอกของสายราและ growth rate ของเชื้อ *P. insidiosum* หรือไม่

8. วิธีการทดลอง

8.1 Serum samples

A total of 37 pythiosis serum samples were used in a performance comparison of serodiagnostic tests. These sera were collected from vascular (n=32) and cutaneous (n=5) pythiosis patients diagnosed based on at least one of the following criteria: (i) successful isolation of *P. insidiosum* from infected tissue (n=8) [30], or (ii) successful detection of anti–*P. insidiosum* antibodies in serum samples (n=37) by at least one of the following established serodiagnostic tests: ID, ELISA, HA or ICT [31–34]. Control samples (n=248) included: (i) 200 serum samples from healthy blood donors who came to the Blood Bank Division, Department of Pathology, Ramathibodi Hospital; (ii) 10 serum samples from thalassemic patients without clinical evidence of pythiosis; (iii) 13 serum samples from patients with positive antinuclear antibody (n=10) or rheumatoid factor (n=3); and (iv) 25 serum samples from patients positive for other infectious

diseases (aspergillosis, 3; zygomycosis, 2; candidiasis, 1; histoplasmosis, 1; cryptococcosis, 1; anti-human immunodeficiency virus antibody, 3; syphilis, 3; anti-hepatitis B virus, 7; and anti-hepatitis C virus, 4). Pooled positive and negative serum samples were prepared and used as an internal control in all assays. All serum samples were kept at -20°C until used (the maximum length of time sera were stored at -20°C prior to testing was ~5 years).

8.2 Microorganisms and culture condition

Thirty-five clinical (n=31) and environmental (n=4) isolates of *P. insidiosum* were recruited for genomic DNA (gDNA) preparation. Identity of all *P. insidiosum* isolates was confirmed by culture identification and zoospore induction. Forty-eight culture-proven isolates of various fungi (**Table 1**) from the Clinical Microbiology Laboratory, Department of Pathology, Faculty of Medicine, Ramathibodi Hospital, Mahidol University, were also collected for gDNA preparation (**Table 1**). All microorganisms were maintained on Sabouraud dextrose agar until use.

8.3 Antigen preparation (CFA and SABH)

The *P. insidiosum* microorganism 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.

Table 1: *Pythium insidiosum* isolates (n=35) and fungal controls (n=48) used for genomic DNA preparation in this study.

Microorganism	Number of isolates
Pythium insidiosum [a]	35
Cryptococcus neoformans	2
Penicillium marneffei	1
Candida species [b]	12
Aspergillus species [c]	3
Mucor species	3
Rhizopus species	2
Absidia species	1
Saksenaea species	1
Conidiobolus species	1
Basidiobolus species	1
Microsporum gypseum	1
Trichophyton species [d]	4
Trichosporon species [e]	2
Trichoderma species	1
Fusarium species	4
Curvularia species	1
Geotrichum species	1
Rhodotorula species	1
Torulopsis glabrata	2
Acremorium species	2
Scedeosporium apiospermum	1
Exophiala jeanselmei	1

Footnote:

[a] *P. insidiosum* isolated from patients with vascular pythiosis (n=14), patients with ocular pythiosis (n=10), patients with cutaneous pythiosis (n=3), patients with other forms of pythiosis (n=2); isolated from animals (n=2); and isolated from environment (n=4)

[b] *Candida* species: *C. albicans* (n=2), *C. tropicalis* (n=2), *C. parapsilosis* (n=2), *C. quilliermondii* (n=2), *C. rugosa* (n=1), *C. lusitaniae* (n=1), *C. laurenttii* (n=1), and *C. dublinensis* (n=1)

[c] *Aspergillus* species: *A. flavus* (n=1), *A. terrcus* (n=1), and *A. fumigatus* (n=1)

[d] *Trichophyton* species: *T. rubrum* (n=3), and *T. mentagrophytes* (n=1)

[e] *Trichosporon* species: *T. asahii* (n=1), and *T. mucoides* (n=1)

8.4 SDS-PAGE and Western blot analysis

SDS-PAGE and Western blotting analysis were performed according to the procedures described previously [19]. 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 x g for 5 min. The proteins in the supernatant were separated at 100 V in an SDSpolyacrylamide 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% H2O2. The reactions were stopped by immersing the membranes in distilled water. Membranes in which the color developed were immediately photographed.

8.5 DNA extraction

The microorganisms were sub-cultured on Sabouraud dextrose agar once a month. For preparation of large-scale gDNA, 10 small plugs (0.5 x 0.5 cm) of 7-day old mycelium-attached agar were transferred to 100 ml of Sabouraud dextrose broth, and incubated with shaking at 37°C for 10 days. For preparation of small-scale gDNA, *P. insidiosum* hyphae were cultivated in a Petri dish with 10 ml of Sabouraud dextrose broth and incubated at room temperature for 10 days. All mycelial mats were harvested, washed with distilled water, filtrated through filter paper (Whatman No.1), and stored at -30°C until use.

The harvested mycelial mats of *P. insidiosum* were subjected to gDNA extraction by three different protocols: conventional-extraction, rapid-extraction, and salt-extraction. The conventional-extraction protocol was performed using the modified method of Jackson *et al.* [35–37]. Briefly,

200-1,000 mg of mycelia were ground to fine powder in the presence of liquid nitrogen. The powder was transferred to a 50-ml conical tube. Twenty ml of extraction buffer [100 mM Tris-HCl (pH 8.0), 100 mM EDTA, 250 mM NaCl, 40 µg/ml proteinase K and 1% SDS] per 1 g of the mycelial power was added to the tube. The mixture was incubated at 55°C overnight. DNA was extracted with phenol:chloroform:isoamyl alcohol (25:24:1), followed by chloroform:isoamyl alcohol (96:4). An equal volume of ice-cold isopropanol was used to precipitate DNA. The DNA pellet was washed with 70% ethanol, air dried, dissolved in 500 µl TE buffer, and treated with RNase for 1 hr (final concentration, 50 µg/ml). The DNA sample was further extracted with chloroform:isoamyl alcohol (96:4), and precipitated with 0.1 volume of 3M sodium acetate and one volume of ice-cold isopropanol. The pellet was washed twice with 70% ethanol, air dried, and dissolved in 50-100 µl TE buffer.

The rapid-extraction protocol was performed according to the method of Vanittanakom [38]. Briefly, 0.5 ml of lysis buffer [1.5%SDS and 0.25 M Tris (pH 8.0)] was added to 50-100 mg of harvested mycelia, boiled for 30 min, and vortexed for 2 min. DNA was then isolated with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) and precipitated with absolute ethanol. A DNA pellet was air dried and dissolved in 50 µl TE buffer.

The salt-extraction protocol was performed using the modified methods of Aljanabi *et al.* [39]. Mycelial mats (50-100 mg) were transferred to a sterile microtube containing glass beads (diameter, 710-1,180 µm; Sigma) and 400 µl of the salt homogenizing buffer [0.4 M NaCl, 10 mM Tris-HCl (pH8.0), 2 mM EDTA] and homogenized using TissueLyzer MM301 (Qiagen, Germany) with the following setting: 2 min at 30 Hz. Forty-five µl of 17 % SDS and 8 µl of 20 mg/ml proteinase K were added to the cell lysate, gently mixed, and incubated at 56 $^{\circ}$C for 2 hr (or overnight). Next, 0.3 ml of 6 M NaCl was added to the sample. The mixture was vortexed for 30 sec, and centrifuged (10,000 x g) for 30 min. Supernatant was collected, mixed with an equal volume of isopropanol, and kept at -20 $^{\circ}$C for 1 hr. The sample was centrifuged (10,000 x g) at 4 $^{\circ}$C for 20 min. The pellet was washed with 70% ethanol, air dried, and dissolved in 100 µl sterile water.

Concentration and purity of all DNA samples was estimated by measurement of optical density at 260 nm and 280 nm wavelengths using a NanoDrop® 2000 spectrophotometer (Thermo Scientific) and by fluorescence-based measurement using a Qubit® 2.0 fluorometer

(Invitrogen). DNA integrity was evaluated by 1% agarose gel electrophoresis. All DNA samples were stored at -30 °C until use.

8.6 RNA Extraction

The microorganism was cultured in a Petri dish containing 10 ml of Sabouraud dextrose broth and incubated at two different temperatures, 28 °C (room temperature; RT) and 37 °C (body temperature; BT), for one week. Mycelia, from each growth condition, was rinsed briefly with pre-warmed (28 or 37 °C) sterile water. The mycelia was transferred to a 2-ml microcentrifuge tube (~500 mg of hyphae/tube), and re-incubated at previous growth temperatures for 24 hours. Growth conditions were identical, except for temperature. Microcentrifuge tubes containing mycelia were then snap-frozen in liquid nitrogen, and stored at -80 °C until use. The frozen hyphae were disrupted two times with glass beads (diameter, 710-1,180 µm, Sigma), using TissueLyzer MM301 (Qiagen, Germany), with the following setting: 2 min at 30 Hz. Total RNA was extracted using Trizol Reagents (Invitrogen, Carlsbad, CA), and then purified using an RNeasy Mini kit (Qiagen), according to the manufacturers' protocols. The obtained total RNA was treated with DNAfree Turbo (Ambion) to remove contaminating DNA. The total RNA concentration and purity were measured using spectrophotometry (NanoDrop 2000, Thermo scientific), and the integrity was evaluated by agarose gel electrophoresis. The total RNA was stored at -80 °C until use for the mRNA purification step.

8.7 Direct DNA Sequencing

PCR products were purified using the NucleoSpinR Gel and PCR clean-up kit (NucleoSpin, USA). Direct sequencing of PCR products was performed using the BigDye Terminator (version 3.1) cycle sequencing kit (Applied Biosystems). By using a forward and/or reverse primers of choice, automated sequencing was carried out using an ABI 3100 genetic analyzer (Applied Biosystems), and the sequences was analyzed using the Applied Biosystems sequencing software. For sequence homology analysis, each sequences obtained were blasted against the NCBI nucleotide database (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

8.8 cDNA library construction and 454 pyrosequencing

A total of 200 ng of poly-A mRNA sample was isolated from the total RNA, using an Absolutely mRNA Purification kit (Stratagene), and fragmented in 1x fragmentation buffer (0.1 M

$ZnCl_2$, 0.1 M Tris-HCl, pH7.0) at 70 °C for 30 sec. The reaction was stopped by adding 2 μl of 0.5 M EDTA and 28 μl of 10 mM Tris HCl, pH7.5. The mRNA sample was cleaned using Agencourt RNAClean reagent (Beckman Coulter), washed with 200 μl of 70% EtOH, air dried and eluted in 20 μl of 10 mM Tris HCl, pH7.5. Fragmented mRNA samples were separated by RNA 6000 Pico kit on the Agilent 2100 bioanalyzer (Agilent Technologies). Fragmented mRNA samples were converted to double-stranded cDNA with the cDNA Synthesis System Kit (Roche Applied Science) using random primers and AMV Reverse Transcriptase. A cDNA library for 454 pyrosequencing was prepared according to the cDNA Rapid Library Preparation protocol (Roche Applied Sciences). The cDNA library was amplified in emulsion PCR and subject to pyrosequencing on a picotitre plate of the Genome Sequencer (GS) FLX Titanium platform (Roche Applied Sciences).

Assembly of 454 GS-FLX sequences was performed using iAssembler [40]. FASTA and associated quality files were firstly preprocessed for the removal of sequence adapters. Cleaned files were passed to iAssembler to obtain the unique sequences (unigenes). iAssembler is a standalone Perl package, which assemble ESTs using repetitive cycles of MIRA, followed by CAP3 assembly. All unigenes were conceptually translated into putative proteins, using the ESTscan program [41]. All the unigenes and predicted proteins were annotated using BLASTP algorithm and the NCBI's non-redundant protein database (E-value cut-off $\leq 1e^{-4}$).

8.9 Phylogenetic analysis

The analysis was performed on the Phylogeny.fr platform [42]. First, sequences were aligned with MUSCLE (v3.7), configured for highest accuracy [43]. After alignment, ambiguous regions (gaps or poorly aligned) were removed with Gblocks (v0.91b) [44]. The phylogenetic tree was reconstructed using the maximum-likelihood method implemented in the PhyML program (v3.0), and the reliability of internal branches was assessed using the aLRT test [45,46]. The graphic representation of the phylogenetic tree was produced with Dendroscope (v3.2.2) [47].

8.10 RACE PCR

The RACE PCR technique is based on oligo-capping and RNA ligase-mediated (RLM) RACE methods (50-51). The GeneRacer™ method involves selectively ligating an RNA oligonucleotide (GeneRacer™ RNA Oligo) to the full-length 5' ends of decapped mRNA using T4

RNA ligase. cDNA was generated via RNA ligase-mediated rapid amplification of 5' and 3' cDNA ends (RLM-RACE PCR) (GeneRacer™, Invitrogen). RNA ligase-mediated rapid amplification of 5' and 3' cDNA ends (RLM-RACE) was performed by mRNA dephosphorylation, mRNA cap removing, then ligation of decapped RNA with the RNA oligo called GeneRacer (known sequence for GeneRacer™ 5' primer and GeneRacer™ 5' Nested primer). Reverse transcription for cDNA synthesis was performed by GeneRacer™ oligo dT primer (poly-T with known adaptor sequence for GeneRacer™ 3' primer and GeneRacer™ 3' Nested primer). RLM-RACE PCR was performed to obtain glucanase sequence in both 5' and 3' end by using GeneRacer™ oligo dT primers and glucanase gene specific primers, then all products was sequenced.

Table 2: Glucanase gene of other microorganisms

Species	Accession Number
Erwinia chrysanthemi	Y00540.1
Bacillus cellulase	M14781.1
Acidothermus cellulolyticus	U33212.1
Cryptococcus flavus	D13967.1
Humicola insolens	X76046.1
Trichoderma reesei	M19373.1
Saccharomyces cerevisiae	S52935.1
Yarrowia lipolytica	Z46872.1
Paracoccidioides brasiliensis	U26160.3
Candida albicans	X56556.1
Saccharomyces cerevisiae	M34341.1
S. cerevisiae	Z46870.1
Cochliobolus carbonum	AF229446.1
Agaricus bisporus	S72325.1
Debaryomyces occidentalis	Z46871.1
Phytophthora ramorum	Pr41489
P. ramorum	Pr39341
P. ramorum	Pr71348
P. ramorum	Pr84167
Hyaloperonospora parasitica	806582
H_parasitica	806581
Pythium ultimum	PYU1G011796
P. ultimum	PYU1G011815
Phytophthora infestans	PITG09798

Species	Accession Number
P. infestans	PITG09799
P. infestans	PITG10218
P. infestans	PITG04615.1
P. infestans	PITG10217.1
Saprolegnia parasitica	SPRG13455
Phytophthora sojae	Ps109097.1
P. sojae	Ps109098.1
P. sojae	Ps119144.1
P. sojae	Ps116268.1
P. sojae	Ps108626.1
P. sojae	Ps145637.1
Phytophthora cinnamomi	AJ938158.1
Aspergillus niger	XM001398831.2
Schizosaccharomyces japonicus	XM002176034.1
Aspergillus oryzae	XM001820049.2
A. flavus	XM002374295.1
Penicillium chrysogenum	XM002562056.1
Candida tropicalis	XM002549991.1
C. dubliniensis	XM002416906.1
Zygosaccharomyces rouxii	XM002498626.1
Penicillium marneffei	XM002150320.1
Pichia angusta	AEOI01000012.1
Cryptococcus neoformans	AJ486863.1
C. gattii	XM003194284.1

8.11 Adaptor PCR

Adaptor PCR used in this study is a modified method of Siebert et al. (40). Briefly, genomic DNA was digested separately with several blunted-end restriction enzymes (*Dra*I, *Eco*RV, *Pvu*II, *Sca*I, *Sma*I, *Nru*I, *Stu*I, *Hpa*I, *Nae*I) to get various sizes of DNA fragments before ligation with the

Adaptor sequence. The adaptor-ligated gDNA fragments was subject to the first-round adaptor PCR amplification using the outer adaptor and the outer gene-specific primers (Table 1). If an expected PCR product was faint or there were multi products, the second round of adaptor PCR was performed using the inner adaptor primer and the inner gene-specific primer (Table 1).

8.12 Polymerase Chain Reaction

The PCR amplifications were performed in a 25-µl mixture comprising 100 ng gDNA template, 10mM each primer of choice, 1.5 mM $MgCl_2$, 0.2 mM dNTP mixture (Promega, USA), and 0.5 UI Taq-polymerase (Fermentas, USA) in 1X Taq-polymerase buffer. Amplification reactions were carried out in a Mastercycler-Pro thermal cycler (Eppendorf, USA) with the following setting: pre-denaturation at 94 °C for 3 min, 25 cycles of denaturation at 94 °C for 45 sec, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min, followed by the final extension at 72 °C for 7 min. A negative control (no template) was included in each round of PCR assays. The GeneRuler™ 100-bp plus DNA ladder (Fermentas, USA) was used as the molecular markers. Size and amount of each PCR product were analyzed by 1% agarose gel electrophoresis, and image captured by Molecular Imager® Gel Doc™ XR+ (Bio-Rad, USA).

8.13 Relative quantitative Realtime PCR

P. insidiosum was cultured in various stages of growth development, under stress condition, carbon source depletion, and zoospore production. RNA extraction was performed using Trizol® reagent (Invitrogen) and bead beater. DNA contamination was treated with DNase-I (Qiagen) while RNA was subjected to clean up using RNeasy kit (Qiagen). cDNA was synthesized with random primer in order to use as realtime PCR template. For realtime PCR of the *Pythium* glucanase gene, primers was designed using the Primer-BLAST program. The actin gene, a housekeeping gene, was used as internal control (the primers: ActA-F 5'-CGGCTCCGGCATGTGCAAGGC-3' and ActA-R 5'-GCCGGCACGTTGAACGTCTC-3'). Conditions for the realtime PCR method was optimized. PCR products was detected using SsoFast™ EvaGreen® Supermix (Bio-Rad). The glucanase transcripts was compared to the level of transcription of internal control.

Table 3: List of the primers used in this study

Primer	Sequence	Description
GeneRacer™ 5'	5'-CGACTGGAGCACGAGGACACTGA-3	RACE PCR
GeneRacer 5' Nested	5'-GGACACTGACATGGACTGAAGGAGTA-3′	RACE PCR
GeneRacer 3'	5'-GCTGTCAACGATACGCTACGTAACG-3′	RACE PCR
GeneRacer 3' Nested	5'-CGCTACGTAACGGCATGACAGTG-3'	RACE PCR
Adaptor L	5'-CTAATACGACTCACTATAGGGCTCGAGCGGCCGCCCGGGCAGGT-3'	Adapter PCR
Adaptor S	5'-PO3-ACCTGCCC-NH2-3'	Adapter PCR
AP1	5'-GGATCCTAATACGACTCACTATAGGGC-3'	Adapter PCR
AP2	5'-AATACGACTCACTATAGGGCTCGAGAGGC-3'	Adapter PCR
Pr61	5'-AACTACGGCAACCTGAAC-3'	Full length
Pr62	5'-TCTTGAGCACCTTGATGG-3'	Full length
Pr67	5'-TTTGCCCCGTGAACAGCAACCTC-3'	Full length
Pr68	5'-GCCCAAGAACATCTCGGACTATG-3'	Full length
Pr70	5'-GTCCTCCGTGACCCAGTTCGC-3'	Full length
Pr71	5'-CACGTGAGCCCGTCCTGTGATC-3'	Full length
Pr72	5'-GTGCCTATGGGCGGCCGATGG-3'	Full length
Pr75	5'-TCAAGCCCTCGCAGATCAACAC-3'	Full length
Pr76	5'-CTTGGGCTCGGCCTTGGCTT-3'	Full length
Pr77	5'-AAGACGTACTACTGGAAG-3'	Realtime PCR
Pr78	5'-CATAAAGTCGAGCCAGAA-3'	Realtime PCR
Pr79	5'-GACCTCCCCTTATCAATCATGGC-3'	Realtime PCR
Pr80	5'-CTTGCACATGCCGGAGCC-3'	Realtime PCR
Dx3	5'-GCGAGTTCTGGCTCGACTTTA-3'	Molecular Diag
Dx4	5'-ACAAGCGCCAAAAAGTCCCA-3'	Molecular Diag

8.14 Transformation of *P. insidiosum*

Protoplast was generated by digesting cell wall of *P. insidiosum* with glucanase and cellulase [Sigma] in 0.8 M Manitol. For the transformation method was performed by electroporation of protoplasts (modified from Weiland et al., 2003 [48]). PEG-mediated transformation [modified from Howard Judelson's protocol] was served as an alternative method if the electroporation failed. The marker (geneticin and hygromycin-B) concentrations was optimized for selection of transformants. This transformation experiment was further used in order to study the function of glucanase by gene silencing and gene over-expression of the *Pythium* glucanase gene.

8.15 Cloning and recombinant protein production

First attempt: The full length sequence (2,229-bp long) of *P. insidiosum* glucanase gene (*PinsEXOI*) has been successfully identified by two methods: RACE PCR and adaptor PCR. For cloning purpose, genomic DNA was isolated from the *P. insidiosum* clinical strain CBS119452, and then amplified by using the forward primer PinEXO1*BamH*I [5'-cagcagggatccctgatccctcgcccac-3'] and the reverse primer PinEXO1*Nco*I [5'-gcggcccatgggttgtactgctggcga-3']. The 2-kb PCR product, which excluded signal peptide sequence, was obtained and then gel purified for the next cloning step.

The purified 2-kb PCR product was cloned to pCR2.1-TOPO, an intermediate cloning plasmid, and transformed to the bacterial host cell DH5α. Transformants were screened using blue/white colony system on X-gal medium plates. Only white colonies (the ones with successful ligation plasmid) were selected for colony PCR (using the primers *PinEXOINco*I and *PinEXOIBamH*I) to check the present of the target gene, as the 2-kb PCR product should be observed on gel. Both *PinsEXO1*-containing pCR2.1-TOPO plasmid and expression vector pRSET-C were digested with two restriction enzymes: *Bam*HI and *Nco*I. Then, the released insert (*PinsEXO1*) were directionally cloned to the linearized expression vector (pRSET-C) before transforming to the bacterial host cell BL21 DE3 pLys for expression.

For a small scale protein expression, the selected bacterial transformant was cultured in 5 ml LB broth under selection of amplicilin and chloramphenicol. Then, 1 mL of this starting culture was transferred to 20 ml of fresh LB broth with these two selection antibiotics and incubated with shaking (200 RPM) at 37°C for 3 hours to reach OD (600nm) ~0.6-0.8 (referred as a before induction culture). IPTG was added to a final concentration of 1 mM, and incubated with shaking (200 RPM) at 37°C for 3 hours (referred as a post induction culture). The before and post induction cultures were boiled to break cells and the cell lysate were subject to SDS-PAGE analysis. Western blot analysis was then performed by using the anti-histidine tag antibody as a recombinant protein-specific probe and alkaline phosphatase conjugate system for signal development.

For a large scale expression, we performed the same steps as for the small scale expression but higher culture volume. The selected bacterial transformant was cultured in 50 ml of LB broth with amplicilin and chloramphenicol selectable markers. Then, 5-10 ml of the culture broth was transferred to 200 ml of fresh LB broth with the selectable antibiotics and cultured with shaking (200RPM) at 37°C for 3 hours to reach 0.6-0.8 OD (600nm). IPTG was added at final

concentration of 1 mM, and incubated (shaken ~200RPM) at 37°C for 3 hours. Cell lysate from the 200-ml post IPTG-induced bacteria was subject to protein extraction using Bugbuster® reagent and 8M urea solution.

Note: The histidine tag was added to the expressed protein for facilitating purification of PinsEXO1. The protein extract in 8M urea solution was added to a Nickel column (Ni-NTA, Qiagen™) and eluted with 3ml each of 25, 50, 100, 250, and 500 mM imidazole in 8M urea buffer. All eluted samples were tested by SDS-PAGE and western blot analyses, as described above. The 500-mM imidazole elution fraction showed highest protein concentration. Recombinant PinsEXO1 protein (in the 500mM immidazole fraction) was dialyzed against PBS for two nights to correct protein folding before rechecking the protein again by SDS-PAGE and Western blot (Figure 3).

Second attempt: Seemingly-degraded expressed protein products from the first attempt of cloning and expression suggested us to second attempt experiment with some modification. This experiment changed the expression vector from pRSET to pET-28b. In addition, we also changed *E. coli* host strain from BL21 DE3 pLys to 2 other strains, namely, Rosetta™ and Rosetta-gami2. Rosetta™ host is BL21 derivative designed to enhance the expression of eukaryotic proteins that contain codons rarely used in *E. coli*. Rosetta-gami2 host enhances disulfide bond formation in the cytoplasm when heterologoous proteins are expressed in *E. coli*. The primers from cloning were HypoEnz_F_NdeI [5'-GGGACAGCATATGCAACAAGCAGCAGCAATTC-3'] and HypoEnz_R_EcoRI [5'- CCAGAATTCCTATGGGCGGCCGATGGGG-3']. The steps for cloning and expression were similar to those for the first attempt experiment.

8.16 Epitope prediction and biotin-labelled peptides

A partial sequence of the putative exo-1,3-β-glucanase encoding gene (*PinsEXO1*) of *P. insidiosum* (accession number: GU994093.1) [49] was BLAST searched against a local transcriptome database of this pathogen (unpublished data). A resulting longer gene sequence was subject to protein translation using the ESTscan program [41], and then, epitope prediction using the PREDITOP program (the program is based on turn predictions, and the peptides chosen are invariably very hydrophilic) [50]. Peptides with predicted antigenic determinant were synthesized (>95% purity) and biotin-labelled by PEPNOME limited (China).

8.17 Rabbit ploYclonal antibody production

The synthetic glucanase peptide was conjugated with the keyhole limpet hemocyanin (KLH) carrier protein at the 1:1 ratio in PBS. The synthetic glucanase peptide was mixed with an adjuvant before using for the anti-serum production in a female New Zealand White rabbit. The rabbit was subcutaneously immunized with the peptides, for 5 times, over the course of 42 days. Before the antigen injection at day 7 and 14, and after the antigen injection at day 42, blood was drawn from rabbit auricular veins. Serum was separated from the obtained blood by centrifugation, and kept at -80 °C until used.

8.18 Immunohistochemical staining protocol (IHC)

A tissue section slide was deparaffinized in xylene, rehydrated in ethanol, and washed in phosphate-buffered saline (PBS; pH 7.4). The tissue slide was incubated with TE buffer (pH 9.0) at 95 °C (in water bath) for 40 minutes. To reduce nonspecific background staining due to endogenous peroxidase, the slide was incubated in 10% H202 for 10 minute, and then washed in PBS. Two hundreds µl of rabbit anti-glucanase peptide, elicitin, or CFA serum was added to the pre-treatment tissue section slide, and stored overnight in a moisture chamber at 4 °C. The slide was washed 3 times with PBS (5 minutes each). The tissue section slide was incubated with a secondary antibody conjugated with horseradish-peroxidase (Thermo Scientific) for 30 minutes. Color development was performed by incubating the slide with DAB for 5 minutes. Finally, the tissue section slide was counter stained with hematoxylin for 5 minutes, before examining the stained slide under a light microscope.

8.19 Immunofluorescent staining protocol (IF)

A tissue section slide was deparaffinized in xylene, rehydrated in ethanol, and washed in PBS (pH 7.4). The tissue section slide was treated with 3.7% formaldehyde, incubate for 30 minutes at 37 °C, and washed 3 times with PBS. Cell or hyphae was permeabilized by adding 0.2% Triton X-100 in PBS and incubated for 5 minutes at room temperature. To reduce nonspecific background staining due to endogenous peroxidase, the slide was incubated in 3% H202 for 5 minute, and then washed in PBS. The permeabilized cell/hyphae was blocked with 6% Bovine serum albumin (BSA) in order to minimize nonspecific binding. The cell/hyphae was stained with 200 µl rabbit anti-glucanase peptide, elicitin, or CFA serum, incubated overnight in a moisture chamber at 4 °C. The slide was washed 3 times with PBS, and incubated the

secondary antibody conjugated with AlexaFlour-568 for 2 hours at room temperature. Finally, the slide was washed deionized water, and mounted with glycerol. The stained slide was examined under a fluorescent microscope.

8.20 CFA-based ELISA

CFA-based ELISA was performed, using the method of Chareonsirisuthigul *et al* [51], with some modifications. Briefly, a 96-well polystyrene plate (Costar, USA) was coated with 100 µl of CFA (5 µg/ml) in 0.1 M carbonate buffer (pH 9.6) and incubated at 4°C overnight, and then washed four times with phosphate buffer solution (pH 7.4) containing 0.5% Tween20 (PBS-T). The CFA-coated plate was blocked with 100 µl of casein buffer [1% casein in phosphate buffer solution (pH 9.6)] at 37°C for 1 hr, and washed four times with PBS-T. A test serum sample diluted 1:800 in the casein buffer was added to each well (100 µl/well), incubated at 37°C for 2 hr, and washed four times with PBS-T. The positive and negative control sera were tested in parallel. Each serum sample was tested in duplicate. Horseradish peroxidase-conjugated goat anti-human immunoglobulin G (Jackson immuno research, USA) diluted 1:100,000 in the casein buffer was added to each well (100 µl/well), incubated at 37°C for 1 hr, and washed four times with PBS-T. Freshly-prepared chromogen [20 µl of 0.6% TMB and 1 ml of 0.009% hydrogen peroxide in acetate buffer solution (25 µmol/ml)] was added to each well (100 µl/well) and incubated at room temperature for ~5 min. The enzymatic reaction was stopped with 100 µl of 0.3 N sulfuric acid.

8.21 Peptide-based ELISA

A 96-well NeutrAvidin coated plate (Pierce, USA) was coated with 100 µl of the biotin-labelled peptide-A or peptide-B (5 µg/ml) in the carbonate buffer and incubated at 4°C overnight. The coated plate was then washed four times with PBS-T. A test serum sample diluted 1:1,600 in the casein buffer was added to each well (100 µl/well), incubated at 37°C for 2 hr, and washed four times with PBS-T. The positive and negative control sera were tested in parallel. Each serum sample was tested in duplicate. Horseradish peroxidase-conjugated goat anti-human immunoglobulin G (Jackson immuno research, USA) diluted 1:2,000 in the casein buffer was added to each well (100 µl/well), incubated at 37°C for 1 hr, and washed four times with PBS-T.

The chromogen solution (prepared as above) was added to each well (100 µl/well) and incubated at room temperature for ~5 min. The enzymatic reaction was stopped with 100 µl of 0.3 N sulfuric acid.

8.22 Measurement of ELISA signal and statistical analysis

Optical density (OD) of each sample was measured using an Infinite 200 Pro ELISA reader (Tecan, Austria) at the wavelength 595-nm. Mean OD value of each sample was corrected for the OD of the buffer control (the casein buffer). The OD value of each serum sample was divided by the OD value of the same negative control serum, to obtain an ELISA value (EV). Sensitivity [which is “True positive (TP) / (False negative (FN) + TP) x 100”], specificity [which is “True negative (TN) / (False positive (FP) + TN) x 100”], accuracy [which is “(TP + TN) / (TP + TN + FP + FN) x 100], mean value of EV, and standard deviation (SD) were calculated using the Microsoft EXCEL2013 program.

8.23 Immunodiffusion

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

8.24 Immunochromatography

ICT strip was prepared as described [33]. The ICT was tested in duplicate with 100 µl of diluted test or control serum (pooled positive or pooled negative) (1:10,000 in phosphate buffer, pH 7.4) in a 96-well polystyrene plate (Nunc, Roskilde, Denmark). Test and control ICT signals were read within 30 min. The result was considered positive when both test and control signals developed and negative when only the control developed.

8.25 Hemagglutination

HA assay was prepared as described [34]. Sheep red cells were coated with CFA. To perform HA, 25 µl of diluted serum (1:160 in 0.5% bovine serum albumin, 1% normal rabbit serum and 0.1% sodium azide in PBS) was added to each well of 96-well polystyrene plate (Nunc, Roskilde, Denmark). An equal volume of 0.5% CFA-coated sheep red cell suspension was added to each well and gently mixed. For the controls, the CFA-coated sheep red cell suspension was mixed with the pooled positive or negative serum. The plate was incubated for 1 h at room temperature. The presence of agglutination was read as a positive test result, whereas the absence of agglutination was read as a negative test result.

9. ผลการทดลอง

Outline:

9.1 Identification and characterization of exo-1,3-β-Glucanase gene of *P. insidiosum*

9.1.1 Full length sequence ของ glucanase gene

9.1.2 เปรียบเทียบ Glucanase gene sequences ของ *P. insidiosum* กับเชื้อราอื่น

9.1.3 Cellular localization ของ *P. insidiosum* glucanase

9.1.4 Glucanase gene expression ใน developmental stage ต่างๆ

9.1.5 Glucanase function (Biochemical activity และ Morphogenesis)

9.2 Clinical application of *P. insidiosum* exo-1,3-β-Glucanase gene

9.2.1 Moleuclar-based assay

9.2.1a Efficiency comparison of DNA extraction methods

9.2.1b Development of molecular-based diagnostic assay

9.2.2 Serodiagnostic assay

9.2.2a Efficiency comparison of serodiagnostic assays

9.2.2b Development of serodiagnostic assay

9.1.1 Full length sequence ของ glucanase gene

ผู้วิจัยสามารถวิเคราะห์หา full length sequence ของ glucanase gene ของเชื้อ *P. insidiosum* ได้สำเร็จโดยใช้เทคนิค Adaptor PCR และ RACE PCR ทำให้ทราบ gene anatomy ของเชื้อ *P. insidiosum* ซึ่งประกอบด้วย promoter (454bp), 5'UTR, ORF (2,229bp) และ 3'UTR (**Figure 1**) ส่วนของ ORF ได้ถูกนำมา Translate เป็น protein sequence เพื่อ predict โครงสร้างของโปรตีนตัวนี้ และพบว่าโปรตีน

glucanase ของ *P. insidiosum* ประกอบด้วย signal peptide (แสดงว่าโปรตีนเข้าสู่ secretory pathway), Bglc และ X8 domains (แสดงว่าโปรตีนมีหน้าที่เกี่ยวกับ carbohydrate metabolism), และ transmembrane domain (แสดงว่าโปรตีนน่าจะ localize อยู่ที่ cell membrane) (**Figure 2**)

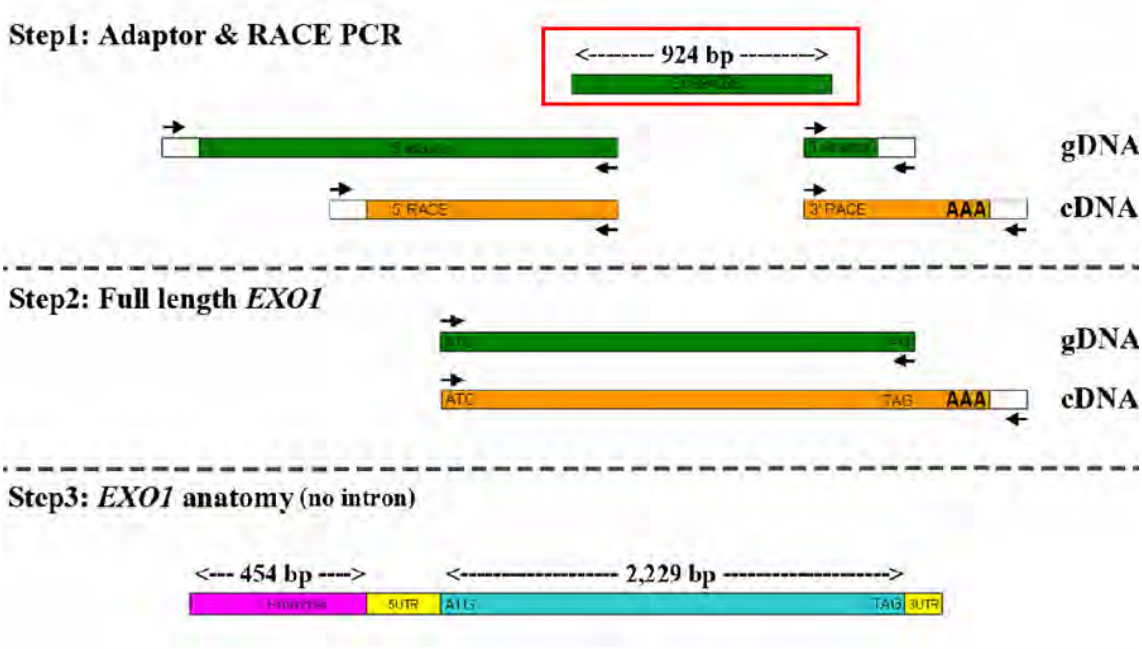


Figure 1. Use of adaptor and RACE PCR to identify the full length sequence of putative exo-1,3-beta glucanase.

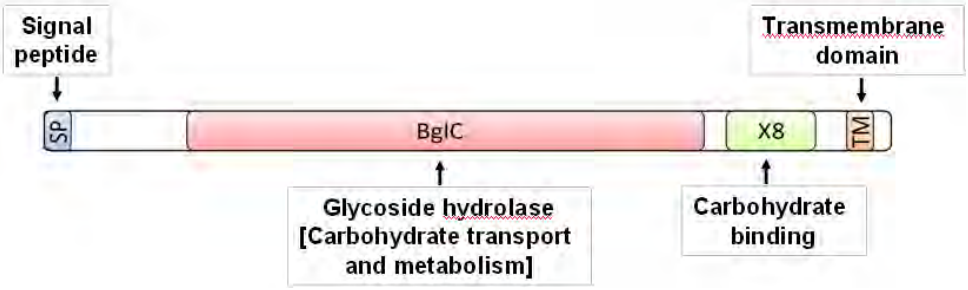


Figure 2. Protein structure of putative exo-1,3-beta glucanase.

9.1.2 เปรียบเทียบ Glucanase gene sequences ของ *P. insidiosum* กับเชื้อราอื่น

ผู้วิจัยได้ PCR และ sequencing ยีน glucanase ของ *P. insidiosum* จำนวน 6 strains เพื่อนำมาวิเคราะห์ทาง phylogenetics ร่วมกับยีนเดียวกันของเชื้อ oomycetes และ fungi ชนิดต่างๆ (โดยใช้

www.phylogeny.fr) พบว่า glucanase genes ของ oomycetes และ fungi แยกออกจากกันเป็นสองกลุ่ม (เนื่องจากมีวิวัฒนาการที่ต่างกัน) และเมื่อดู glucanase ของ *P. insidiosum* ทั้ง 6 strains ก็พบว่าอยู่ด้วยกัน (เพราะเป็น species เดียวกัน) และใกล้กับ *Saprolegnia parasitica* (Spar) ซึ่งเป็น animal pathogenic oomycete มากที่สุด (ส่วนใหญ่ของ oomycetes จะเป็น plant pathogen ยกเว้นบาง species เช่น *P. insidiosum* และ *S. parasitica* ซึ่งเป็น animal pathogen) ดังแสดงใน **Figure 3**

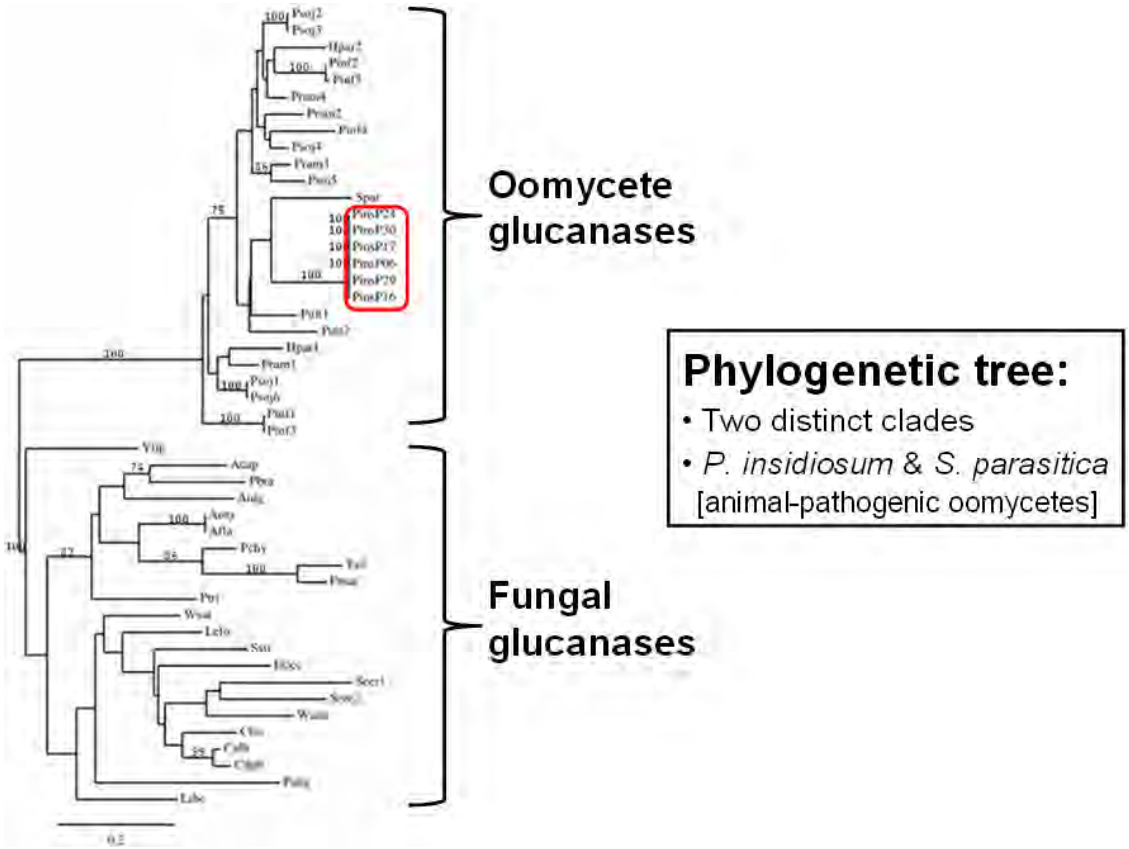


Figure 3. Phylogenetic analysis of exo-1,3-beta glucanase genes of *P. insidiosum*, oomycetes, and fungi.

9.1.3 Glucanase gene expression ใน developmental stage ต่าง ๆ

ผู้วิจัยได้ศึกษา Transcription profile ของยีน glucanase ของ *P. insidiosum* โดยสกัด RNA จากเชื้อจำนวน 3 strains ที่เลี้ยงในอาหารเลี้ยงเชื้อชนิด Sabouraud dextrose agar (SDA) ใน 6 conditions คือ อุณหภูมิห้อง (28 °C หรือ RT) เป็นเวลา 7 วัน, อุณหภูมิร่างกาย (37 °C, BT) เป็นเวลา 7 วัน, อุณหภูมิห้อง เป็นเวลา 14 วัน (old), อุณหภูมิห้อง ในอาหารที่มี glucose เป็น 0.1 เท่า (0.1x), 0.01 เท่า (0.01x), และไม่มีเลย (0x) เมื่อเทียบกับ SDA ปกติ เมื่อเทียบระดับ glucanase transcript กับ RT พบว่า

condition “old”, “BT” และ “0.1x” มี higher expression level ส่วน condition “0.01x” และ “0x” มี lower expression level ดัง **Figure 4** โดยผลที่ได้นั้นสอดคล้องกันทั้ง 3 strains ที่ทำการศึกษา

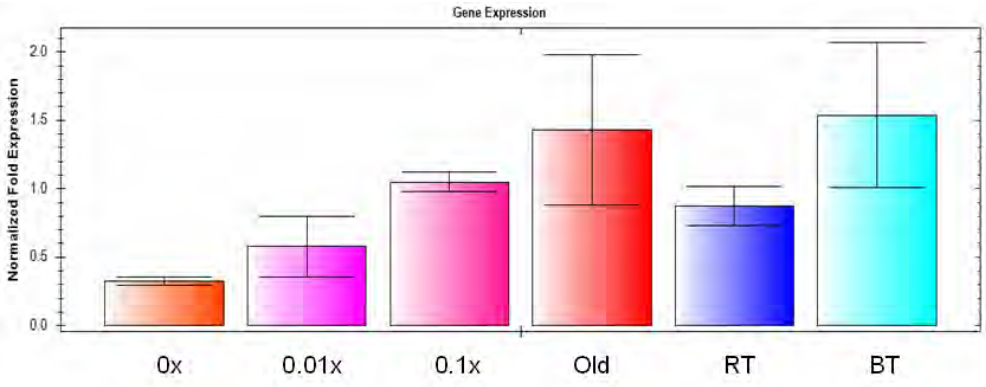


Figure 4. Transcription profile of exo-1,3-beta glucanase gene of *P. insidiosum* (RT, *P. insidiosum* grown at room temperature for 7 days; BT, at body temperature (37C) for 7 days; Old, at room temperature for 14 days; 0.1x, grown in medium with 0.1-fold decreased glucose; 0.01x, 0.01-fold decreased glucose; 0x, no glucose)

9.1.4 Cellular localization ของ *P. insidiosum* glucanase

ในการศึกษา Cellular localization ของ glucanase protein ของเชื้อ *P. insidiosum* จำเป็นต้องอาศัย specific anti-glucanase antibody ผู้วิจัยจึงได้ออกแบบ peptides จำนวน 3 ตัว (peptide#1, 2, 3) ซึ่งทำนายแล้วว่าเป็น B-cell epitopes (โดยใช้โปรแกรม Preditop) เพื่อใช้ในการสร้าง polyclonal antibody จากกระต่าย เมื่อได้ rabbit serum มาก็นำมาทดสอบกับ peptide แต่ละตัว พบว่า serum ดังกล่าวมี immunoreactivity ดีมากต่อ peptide แต่ละตัวโดยเฉพาะอย่างยิ่ง peptide#3 (ทดสอบเทียบกับ pre-immunized serum) ดัง **Figure 5**. จากนั้นเพื่อพิสูจน์ว่า Glucanase peptide กระตุ้น humoral immunity (antibody) ในผู้ป่วย pythiosis หรือไม่ จึงนำ glucanase peptide มาทำปฏิกิริยากับเลือดผู้ป่วยและคนปกติ โดยใช้เทคนิก ELISA (**Figure 6**) พบว่า glucanase ของเชื้อสามารถกระตุ้น antibody ในผู้ป่วยได้

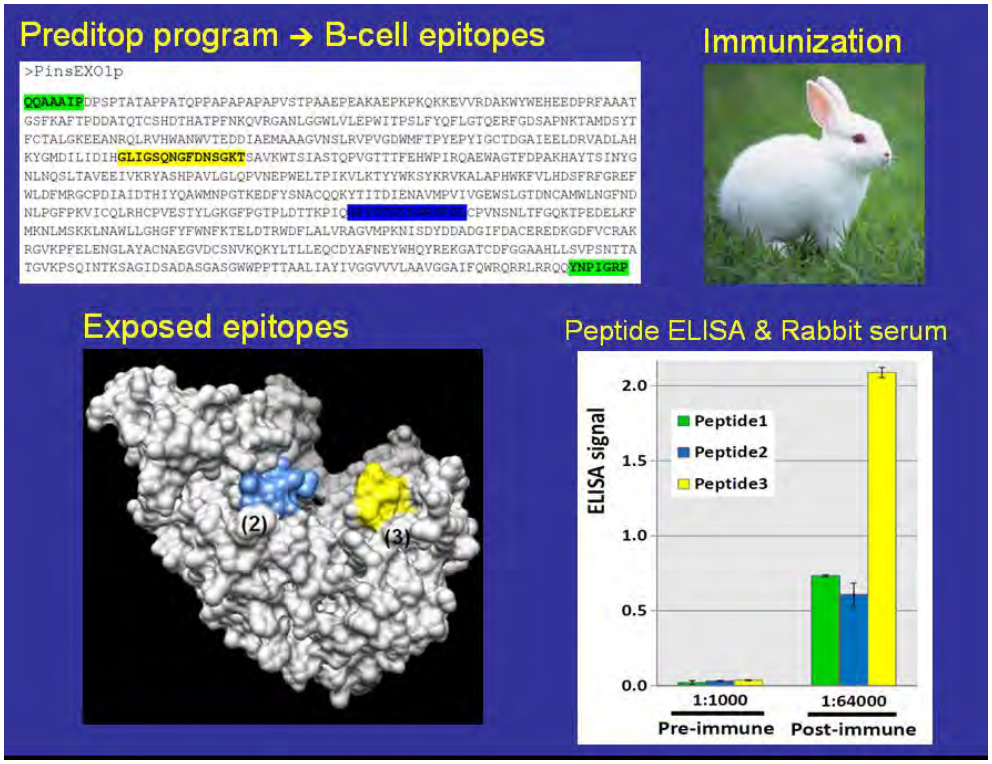


Figure 5. Upper left and Lower left, predicted B-cell epitopes (peptide#1,2,3) of exo-1,3-beta glucanase of *P. insidiosum* are on surface (predicted by homology modeling analysis). Upper right and Lower right, rabbit antiserum (polyclonal antibody) are reactive (by ELISA) against predicted B-cell epitopes of exo-1,3-beta glucanase (peptide#3 is best reactive).

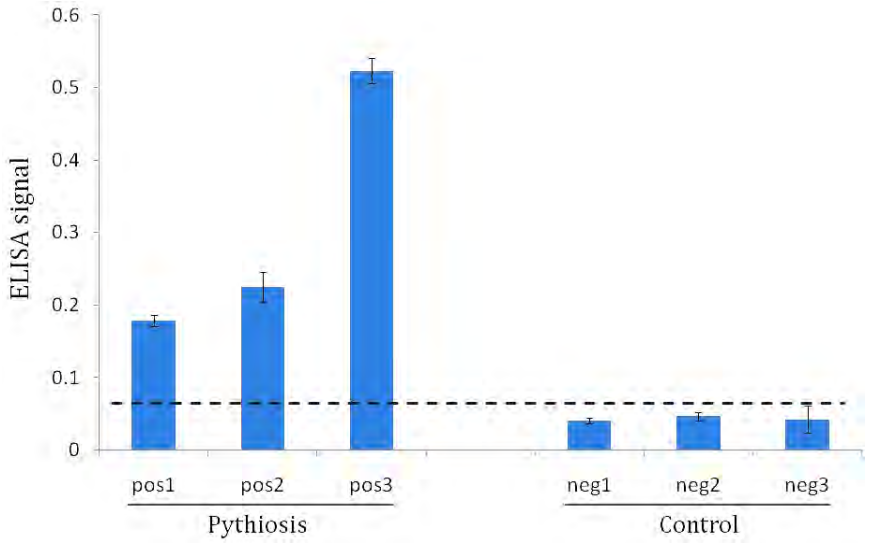
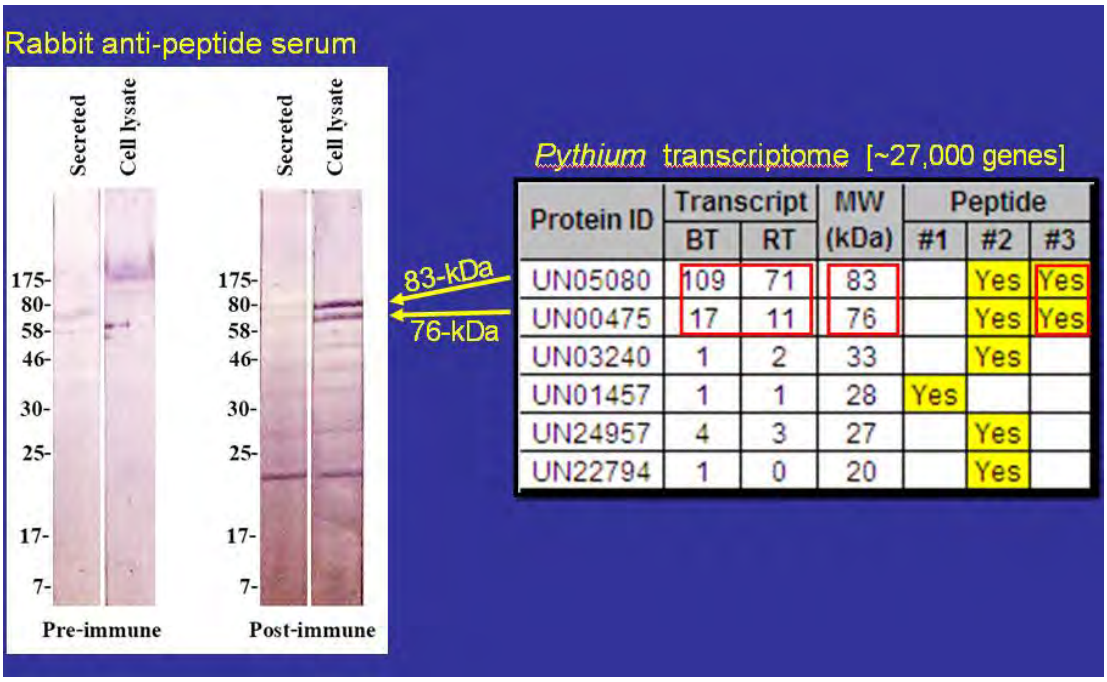


Figure 6. Peptide#3 of exo-1,3-beta glucanase is reactive against serum samples from patients with pythiosis (pos1-3), but not serum samples from healthy blood donor (neg1-3).

จากนั้นผู้วิจัยได้ทดสอบหาว่า glucanase มี cellular localization อยู่ที่ตำแหน่งใดของเชื้อ *P. insidiosum* โดยขั้นแรกได้ทำ Western blot analysis โดยใช้ rabbit anti-glucanase antibody เพื่อทำปฏิกิริยากับ secreted proteins (จาก culture filtrate antigen) และ hyphal proteins (จาก cell lysate) พบว่าเกิด 2 prominent bands ที่ขนาดโมเลกุล 76 และ 83 kDa (Predicted molecular mass ของ glucanase มีขนาด ~80kDa) และ 6 weak bands ที่ขนาดโมเลกุลเล็กลงมา ดัง **Figure 7** จากการวิเคราะห์ predicted proteins จาก Transcriptome ของเชื้อ *P. insidiosum* พบว่ามีโปรตีนจำนวนหนึ่ง (ดังสรุปในในตารางใน **Figure 7**) ที่มี sequence บางส่วนตรงกับ peptide#1, 2 หรือ 3ที่นำมาผลิต anti-glucanase antibody จึงอธิบายการพบ multiple bands ใน Western blot analysis ได้ อย่างไรก็ตามจะเห็นว่า peptide#3 พบเฉพาะในโปรตีนขนาด 83 และ 76 kDa เท่านั้น ขั้นถัดไปผู้วิจัยได้นำ rabbit anti-glucanase antibody ไปย้อม immunostain และพบว่าย้อมติดสีบริเวณ surface ของเชื้อ (**Figure 8**) ดังนั้น cellular localization ของ glucanase น่าจะอยู่ที่ surface ซึ่งสอดคล้องกับการทำนายโครงสร้างโปรตีนของ glucanase ที่ประกอบด้วย signal peptide และ transmembrane domain (**Figure 2**)



Protein ID	Transcript		MW	Peptide		
	BT	RT	(kDa)	#1	#2	#3
UN05080	109	71	83		Yes	Yes
UN00475	17	11	76		Yes	Yes
UN03240	1	2	33		Yes	
UN01457	1	1	28	Yes		
UN24957	4	3	27		Yes	
UN22794	1	0	20		Yes	

Figure 7. Western blot analysis shows rabbit serum immunized against exo-1,3-beta glucanase peptide#1-3. Pre-immunized serum shows no prominent bands, while post-immunized serum shows several bands (Secreted = Secreted proteins or culture filtrate antigens; Cell lysate = hyphal proteins).

Figure 8. Immunohistochemical staining shows rabbit anti-exo-1,3-beta glucanase antibody localizes on surface of *P. insidiosum* hyphae.

9.1.5 Glucanase function (Biochemical activity / Morphogenesis)

เนื่องจากการศึกษา Function ของยีน Glucanase จำเป็นต้องมี DNA transformation method (ใช้กดการแสดงออกของยีน *PinsEXO1* เพื่อดู morphology ที่อาจเปลี่ยนแปลงไป) และจำเป็นที่จะต้องมี recombinant glucanase protein (เพื่อใช้ทดสอบ biochemical activity และใช้ฉีด immunize กระต่ายเพื่อสร้าง polyclonal antibody ต่อ glucanase) ซึ่ง experiments ทั้งสอง เป็นสิ่งที่คณะผู้วิจัยพยายามอย่างมากเพื่อจะทำการทดลองให้ได้ตามมุ่งหวังไว้

ในส่วนของ DNA transformation method นั้น คณะผู้วิจัยได้ทำการทดลอง 2 วิธีการ คือ (i) PEG-mdiated transformation of protoplasts (โดยประยุกต์ protocol ที่ได้มาจาก Dr. Francine Govers, Wageningen University, The Netherlands) ซึ่งต้องอาศัยการผลิต protoplasts จำนวนมาก, และ (ii) Electroporation of zoospore (โดยประยุกต์ protocol ตามวิธีของ Weiland [48]) แต่ประสบข้อจำกัดคือเชื้อ *P. insidiosum* สามารถใช้สร้าง protoplast และ zoospore ได้จำนวนน้อยมาก (น้อยกว่า 1 x 10^6 zoospores หรือ protoplasts) แต่อย่างไรก็ตาม คณะผู้วิจัยก็ได้นำ zoospores/protoplasts ที่ได้ทั้งหมดมาทดลองทั้งวิธี PEG-mdiated transformation และ Electroporation โดยใช้ Plasmid DNA ซึ่งมี selection marker ต่อยา Geneticin (G418) โดยคณะผู้วิจัยพบว่าควรใช้ยา Geneticin ที่ปริมาณ 200 µg/mL ในการคัดเลือก Transfprmants. หลังจากได้พยายามและปรับปรุงวิธีการหลายครั้ง จึงพบว่าวิธี Transformation ดังกล่าวทั้งสองไม่เหมาะสมต่อ *P. insidiosum* เนื่องจากไม่ได้ Transformant (คณะผู้วิจัยมีการวิเคราะห์ และหาแนวทางการแก้ไขปัญหา ดังระบุไว้ในบทวิจารณ์).

ในส่วนของ recombinant glucanase protein เพื่อใช้ทดสอบ biochemical activity และฉีด immunize กระต่ายเพื่อสร้าง polyclonal antibody ต่อ glucanase นั้น คณะผู้วิจัยได้ดำเนินการวิจัยทำ cloning and expression of *Pythium insidiosum* glucanase gene ใน bacterial system. จากการวิเคราะห์โปรตีน

ด้วยวิธี Western blot ทำให้พบปัญหาคือ Glucanase ที่ expressed ได้นั้นไม่ stable และเกิด degraded ถึงแม้จะเปลี่ยน Bacterial host cells หลายชนิดแล้วก็ตาม (วิธีการทดลองข้อ 8.15). จึงเป็นอุปสรรคในการดำเนินการวิจัยขั้นต่อไปเพื่อศึกษา biochemical activity ของ glucanase. (คณะผู้วิจัยมีการวิเคราะห์และหาแนวทางการแก้ไขปัญหา ดังระบุไว้ในบทวิจารณ์). ในส่วนของการสร้าง polyclonal antibody ต่อ recombinant glucanase protein นั้น คณะผู้วิจัยแก้ปัญหาโดยการใช้ synthetic peptides แทน ซึ่งได้ผลการทดลองที่ดี ดังรายงานด้านบน (**Figure 5**).

9.2.1a Efficiency comparison of DNA extraction methods

Quality and quantity of extracted gDNA: Based on fluorescence measurements, the conventional-extraction protocol provided the highest DNA concentration (mean, 323 ng/µl; range, 121-540), followed by the salt-extraction (mean, 114 ng/µl; range, 39-199) and the rapid-extraction (mean, 46 ng/µl; range, 27-79) protocols (**Table 4**). However, the salt-extraction protocol provided the highest total yield (DNA obtained / mass of mycelium) (mean, 145 ng/mg; range, 47-260), followed by conventional-extraction (mean, 100 ng/mg; range, 28-183) and rapid-extraction (mean, 65 ng/mg; range, 39-136) (**Table 4**). A260/A280 ratios of the DNA extracted by the conventional-extraction protocol (~1.8–1.9) were lower than the ratios of the other two protocols (~2.0–2.1) (**Table 4**). DNA integrity was evaluated by agarose gel electrophoresis. High molecular weight DNA (size > 23kb) was observed in all samples prepared by the conventional- and salt-extraction protocols (**Figure 9**). In contrast, this high molecular weight DNA was absent in all samples prepared by the rapid-extraction protocol (**Figure 9**). Degraded DNA (characterized as a smear of low molecular weight material) was minimal in gDNA extracted by the conventional-extraction protocol, noticeable in gDNA extracted by the salt-extraction protocol, and prominent in gDNA extracted by the rapid-extraction protocol.

PCR amplification of target genes: When using the gDNA templates prepared by the conventional-extraction protocol, all primer sets (ITSpy1/2, ITS1/4, Dx3/4, and RXLR1/2; **Table 5)** successfully amplified target genes: *UN04715* (**Figure 10A**), *rDNA* (**Figure 10B and D**), and *EXO1* (**Figure 10C**). When testing gDNA templates prepared by the salt-extraction protocol, all primer sets produced relatively intense bands except for the primers RXLR1/2, which provided faint bands for the T3 and T6 templates (**Figure 10A**). Most of the gDNA templates prepared by the rapid-extraction protocol produced faint bands using primers ITSpy1/2 (**Figure 10D**). The Dx3/4 primers amplified the expected amplicon from five out of seven templates tested (**Figure**

10C); the ITS1/4 primers successfully amplified a product from only the T1 and T2 templates (**Figure 10B**); and the RXLR1/2 primers failed to produce an amplicon from all templates tested (**Figure 10A**).

Table 4: Quality and quantity of extracted genomic DNA of seven *P. insidiosum* strains. Concentrations were estimated by the UV absorbance (NanoDrop spectrophotometer) and the fluorescence-based (Qubit fluorometer) measurements. DNA yields **(**DNA obtained / mass of mycelium) were calculated based on the DNA concentration measured by the Qubit fluorometer.

Strain ID	Reference ID	Source	Concentration (ng/µl): Qubit (NanoDrop)			DNA yield (ng/mg)			A260/280 Ratio		
			Convent extract	Salt extract	Rapid extract	Convent extract	Salt extract	Rapid extract	Convent extract	Salt extract	Rapid extract
T1	CR02	Environment	336 (373)	39 (757)	79 (1,023)	67	47	136	1.9	2.0	2.0
T2	MCC18 / P12	Human (eye)	540 (574)	190 (1,590)	41 (1,330)	149	192	47	1.8	2.1	2.0
T3	Pi-S / P35	Human (leg)	271 (283)	93 (584)	43 (1,994)	122	112	55	1.8	2.1	2.1
T4	CBS777.84	Mosquito	257 (297)	86 (384)	48 (1,181)	101	146	72	1.9	2.1	2.0
T5	CBS673.85	Human (skin)	225 (351)	199 (1,652)	53 (994)	53	260	60	1.9	2.1	2.0
T6	CBS702.83	Horse	510 (557)	52 (418)	27 (360)	183	99	49	1.8	2.1	2.0
T7	P6	Human (leg)	121 (199)	139 (683)	34 (1,646)	28	162	39	1.8	2.1	2.1
Mean	-	-	323 (376)	114 (867)	46 (1,218)	100	145	65	1.8	2.1	2.0
SD	-	-	153 (141)	64 (532)	17 (520)	55	69	33	0.0	0.0	0.1

Table 5: Primer sequences and PCR conditions for amplification of *P. insidiosum* genes (F, forward primer; R, reverse primer).

Target gene	Primer	Sequence	Amplicon size (bp)	Template amount (ng)	Anealling temp (°C)	PCR extension time (sec)
rDNA	ITSpy1 (F) ITSpy2 (R)	5'-CTGCGGAAGGATCATTACC-3' 5'-GTCCTCGGAGTATAGATCAG-3'	233	100	60	30
rDNA	ITS1 (F) ITS4 (R)	5′-TCCGTAGGTGAACCTGCGG-3′ 5′-TCCTCCGCTTATTGATATGC-3′	931	100	55	30
EXO1	Dx3 (F) Dx4 (R)	5'-GCGAGTTCTGGCTCGACTTTA-3' 5'-ACAAGCGCCAAAAAGTCCCA-3'	550	100	57	60
UN04715	RXLR1 (F) RXLR2 (R)	5'-GCCCATGGCCTCTTCGTCCATGAGCTCGCTC-3' 5'-GCGAATTCGCACGACGGGCGGCTCGT-3'	1,575	200	50	120

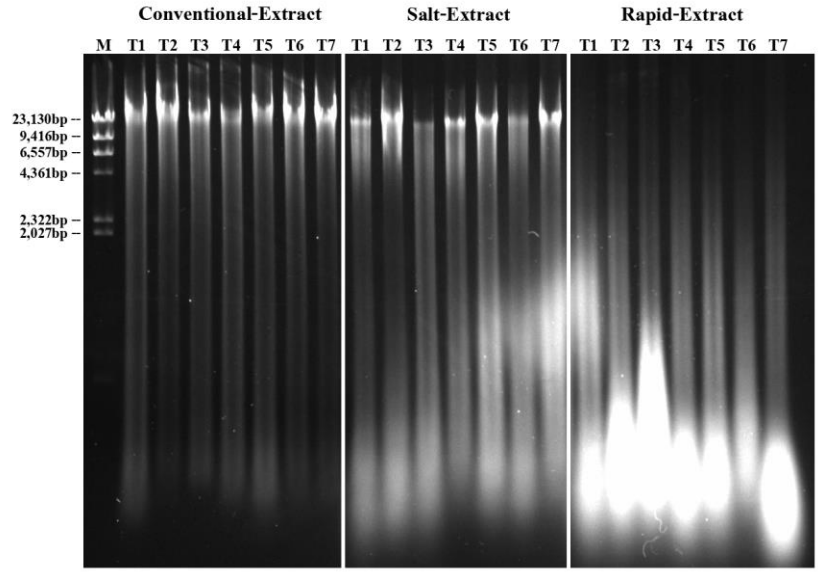


Figure 9: An agarose gel showing genomic DNA of *P. insidiosum* isolate T1-7, prepared by conventional-extraction, salt-extraction, and rapid-extraction protocols (M, molecular weight markers).

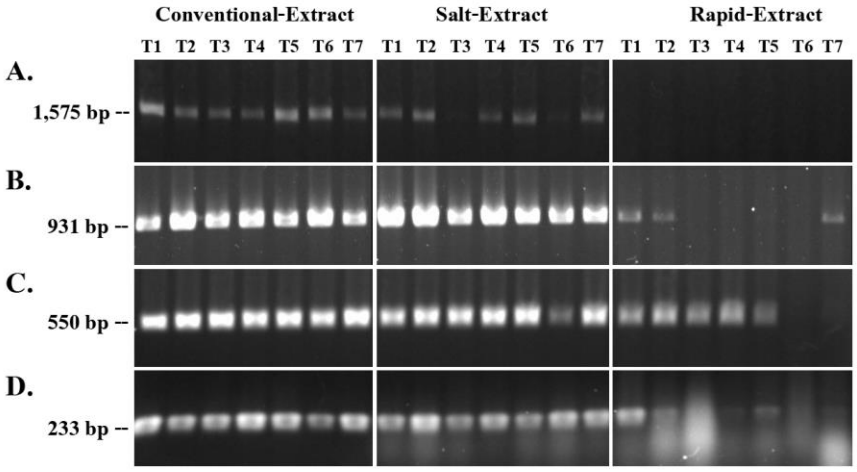


Figure 10: PCR amplification of *P. insidiosum* genes using the DNA templates prepared by conventional-extraction, the salt-extraction, and the rapid-extraction protocols: **A**. the putative RXLR-effector encoding gene (*UN04715*) amplified by the primers RXLR1 and RXLR2; **B**. the ribosomal DNA gene (*rDNA*) amplified by the primers ITS1 and ITS4; **C**. the putative exo-1,3-beta glucanase-encoding gene (*EXO1*) amplified by the primers Dx3 and Dx4; and **D**. *rDNA* amplified by the primers ITSpy1 and ITSpy2.

9.2.1b Development of molecular-based diagnostic assay

The negative control (no template) and the ITS1/4 fungal universal primers were included for a quality check of the gDNA samples from all 83 organisms (**Table 1**). After PCR amplification with the ITS1/4, ITSpy1/2, and newly-designed Dx3/4 (sequences and annealing locations were shown in **Figure 11**) primers, the negative control did not provide any product by gel electrophoresis. The ITS1/4 primers successfully amplified rDNA from all gDNA samples tested (**Table 1**). When amplifying gDNA samples from the fungal controls (n=48; **Table 1**), the ITSpy1/2 primers (which target rDNA) and the Dx3/4 primers (which target *PinsEXO1*) provided no band. Thus, detection specificity of these primer sets were equally 100%.

When testing the gDNA samples, extracted from all *P. insidiosum* isolates, the ITSpy1/2 primers produced an intense band from 29 samples, a very faint band from 3 samples (the isolate P11, NAN06, and SIMI7695.48), and no band from 3 samples (the isolate P8, P13, and P21) (**Figure 12B**). The gDNA prepared from the *P. insidiosum* isolate P1, P22, and Pi-S provided a slightly smaller PCR product (~220 bp), compared to the calculated amplicon size (~230 bp) (**Figure 12B**). However, sequence homology analysis against the NCBI nucleotide database (BLASTN) showed that these amplicons matched the *P. insidiosum* rDNA. Overall, the ITSpy1/2 primers showed the detection sensitivity of 91.4%.

The Dx3/4 primers successfully amplified a single, relatively-intense, 550-bp PCR product from gDNA templates of all 35 *P. insidiosum* isolates tested (**Figure 12A**). Detection sensitivity of the Dx3/4 primers was therefore 100%. BLASTN search against the NCBI nucleotide database matched these amplicons to *PinsEXO1*. The lowest amount of gDNA template required for a successful PCR amplification (limit of detection) of *PinsEXO1*, using the Dx3/4 primers, was 1 ng.

Figure 11: Sequences and annealing locations of the primer Dx3 (first gray box) and Dx4 (second gray box; reverse complement sequence) in reference to the putative exo-1,3-β-glucanase genes from *Pythium insidiosum* (Query sequence; Accession number, GU994093.1) and *Phytophthora infestans* (Subject sequence; Accession number, AF494014.1). The primer Dx3 and Dx4 perfectly anneal to the *P. insidiosum* glucanase gene, but failed to properly anneal to the *P. infestans* glucanase gene. Sequence alignment analysis of the glucanase genes from *P. insidiosum* and *P. infestans* (performing online at http://blast.ncbi.nlm.nih.gov/Blast.cgi) shows E-value of 4e-141, identities of 72%, and gap of 1%.

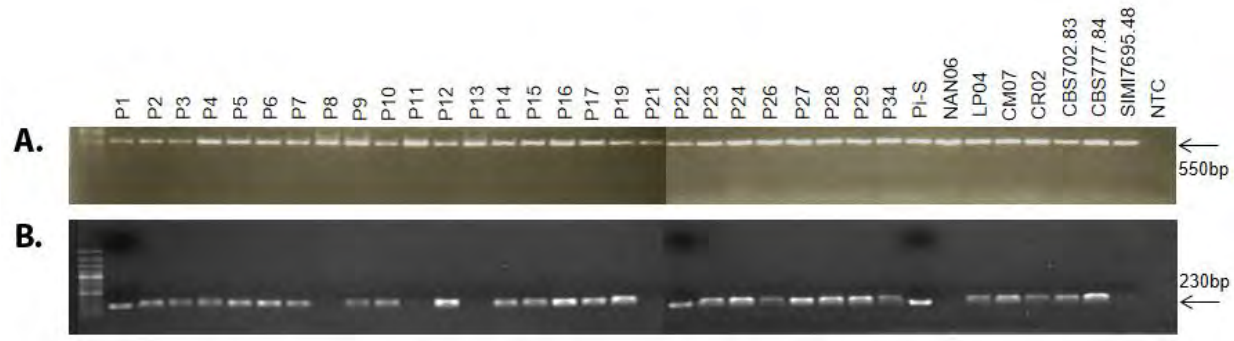


Figure 12: Gel electrophoresis showing the PCR products amplified from gDNA of the 35 *P. insidiosum* isolates by using: (**A**) the Dx3/4 primers, which target the putative exo-1,3-β-glucanase gene of *P. insidiosum* or *PinsEXO1* (Amplicon size, 550bp); and (**B**) the ITSpy1/2 primers, which target the rDNA region of *P. insidiosum* (Amplicon size, 230bp) (NTC, No-template control).

9.2.2a Efficiency comparison of serodiagnostic assays

Development of CFA-based ELISA: Mean EVs of pythiosis (n=37) and control (n=248) sera were 5.1 (range, 2.2–14.6; SD, 2.7) and 1.3 (range, 0.8–2.0; SD, 0.2), respectively. Three cutoff points (EV = 1.7, 1.9 and 2.1) were calculated based on summation of the mean EV of control sera and 2 (mean+2SDs), 3 (mean+3SDs) and 4 (mean+4SDs) SDs, respectively (Figure 1). The mean+2SDs, +3SDs and +4SDs cutoffs provided 96, 99 and 100 % detection specificity, respectively. All the cutoffs provided 100% detection sensitivity. The mean+4SDs EV was selected as the cutoff of ELISA because all pythiosis sera were determined to be positive and all control sera were determined to be negative. EVs of the pooled positive serum from seven independent batch tests provided mean value of 8.6, SD of 0.1 and CV of 1.4%.

Diagnostic performances of ELISA, ID, ICT and HA: ELISA, ID, ICT and HA were compared for performances in detection of anti-*P. insidiosum* antibody using the same set of serum samples. All assays, except HA, were tested against 37 pythiosis and 248 control sera. HA was evaluated with fewer sera (32 pythiosis and 208 control sera) due to unavailability of some samples. To serve as the system controls and ensure accurate result interpretation, all assays were also tested with the same pooled positive and negative sera. For an ID test to be considered positive, 1-3 precipitins of the pooled positive serum formed line(s) of identity with precipitins of a test serum. Detection sensitivity of ELISA and ICT (100%) were markedly higher than ID (68%) and HA (84%) (**Table 6**). Detection specificity of all serological assays were

equivalently high (100%), except HA (82%) (**Table 6**). Accuracies (ability of an assay to provide true positive and true negative results) of ID, ELISA, ICT and HA were 96%, 100%, 100% and 83%, respectively. PPVs of ID, ELISA and ICT were 100%, while PPV of HA was relatively low (42%). All assays showed high NPVs (range, 95-100%; **Table 6**).

Table 6: Performance of ID, ELISA, ICT and HA in the detection of anti-*P. insidiosum* antibody in serum samples (Abbreviations: ID, immunodiffusion; ELISA, enzyme-linked immunosorbent assay; HA, hemagglutination; ICT, immunochromatographic test; PPV, positive predictive value; NPV, negative predictive value).

Assay[a]	**Sensitivity**[b] **(%)**	**Specificity**[c] **(%)**	**Accuracy**[d] **(%)**	**PPV**[e] **(%)**	**NPV**[f] **(%)**	**Assay procedure**	**Interpretation of test result**[g]	**Turnaround time (hr)**
ID	68	100	96	100	95	Single-step	Subjective	24
ELISA	100	100	100	100	100	Multi-step	Objective	3
ICT	100	100	100	100	100	Single-step	Subjective	0.5
HA	84	82	83	42	97	Single-step	Subjective	1

Footnote:

[a] ID, ELISA and ICT were tested with all 37 pythiosis and 248 control sera. Due to limited sample availability, HA was evaluated with 32 pythiosis and 208 control sera.

[b] Sensitivity = true positive/(false negative+true positive) x 100

[c] Specificity = true negative/(false positive+true negative) x 100

[d] Accuracy = (true positive+true negative)/(true positive+true negative+false positive+false negative) x 100

[e] PPV = true positive/(false positive+true positive) x 100

[f] NPV = true negative/(false negative+true negative) x 100

[g] Determination of result via visual assessment is referred to as “subjective” interpretation. Quantification via instrument is referred to as “objective” interpretation.

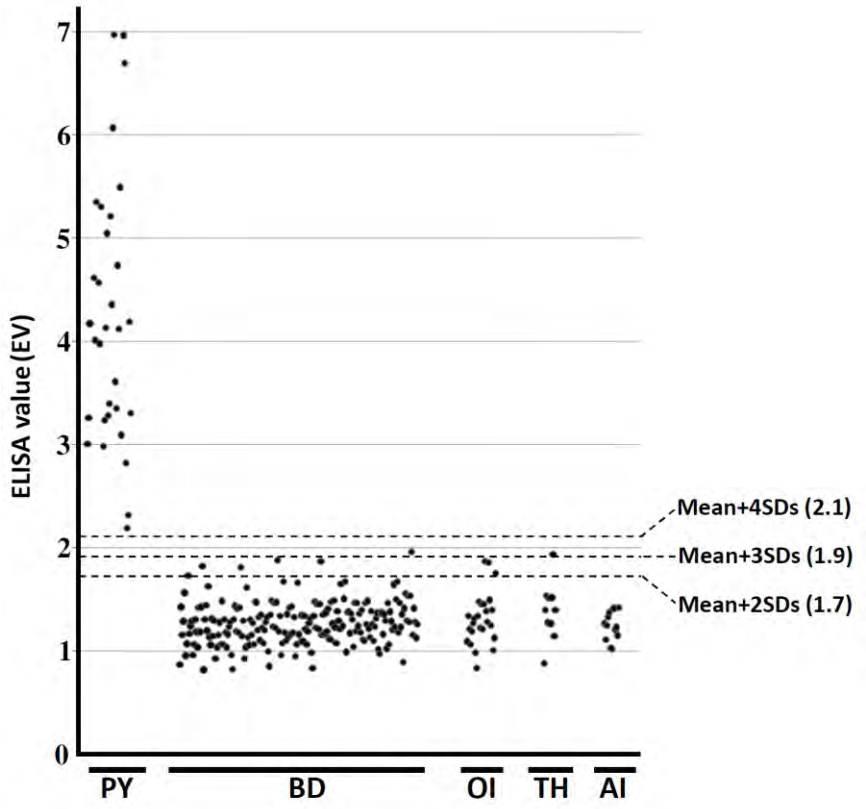


Figure 13: Detection of anti-*P. insidiosum* antibody in sera by ELISA. Three ELISA cutoffs were calculated from the mean value (EV = 1.28) of the control sera (BD, OI, TH and AI) plus 2, 3 and 4 SDs (1 SD = 0.21). Five EVs (range, 8.4–14.6) of the PY serum samples were not shown in the plot. Abbreviations: EV, ELISA value; PY, patients with pythiosis (n=37); BD, healthy blood donors (n=200); OI, patients with other infectious diseases (n=25); TH, healthy thalassemic patients (n=10); AI, patients with autoimmune diseases (n=13).

9.2.2b Development of serodiagnostic assay

Antigenic determinant prediction of *P. insidiosum* glucanase: To obtain a longer sequence, the *PinsEXO1* partial sequence (accession number, GU994093.1) [49] was BLASTN search against our local 454-generated *P. insidiosum* transcriptome database, comprising 26,735 unigenes (unpublished data). The BLAST analysis best matched (E-value, 0.0; identity, 99%; query coverage, 100%) the transcript number UN05080 (submitted to the DNA Data Bank of Japan under accession number FX532070). The corresponding partial *P. insidiosum* glucanase protein (PinsEXO1; 307 amino acids long; accession number, ADI86643.1) identically matched the UN05080-deduced 751-amino acid-long protein (E-value, 0.0; sequence identity, 100%; query

coverage, 100%) (**Figure 14**). The PREDITOP program (Pellequer and Westhof 1993) predicted two antigenic determinants in the UN05080 protein sequence: Peptide-A (GLIGSQNGFDNSGKT) and Peptide-B (GPYGTGTSGPSFGL) (**Figure 14**). Peptide-A and Peptide–B were synthesized (> 95% purity) and biotin-labelled (at the N-terminus).

Diagnostic performance of CFA- and peptide-based ELISAs: To ensure accurate result interpretation, all ELISA analyses included the same positive and negative control sera. Based on all pythiosis sera tested (n=34), mean EVs of the CFA-based, Peptide-A, and Peptide–B ELISA were 145.9 (range, 16.1-236.9; SD, 57.6), 7.7 (range, 2.8–11.7; SD, 2.5), and 2.3 (range, 0.7–5.4; SD, 1.0), respectively. Based on all control sera tested [including serum samples from healthy blood donors (n=56), from thalassemic patients without pythiosis (n=6), from patients with autoimmune diseases (n=11), and from patients with other infectious diseases (n=19)], mean EVs of the CFA, Peptide-A and Peptide–B ELISA were 2.2 (range, 0.0–10.8; SD, 2.1), 0.9 (range, 0.2–2.3; SD, 0.6), and 0.4 (range, 0.1–1.2; SD, 0.3), respectively. Five cutoff points were calculated based on summation of the mean EV of control sera and 2, 3, 4, 5 and 6 SDs, respectively. Each cutoff point provided different detection sensitivity and specificity (**Table 7)**. An optimal cutoff EV was selected based on the highest accuracy obtained (**Table 7**). The summation of mean and 5 SDs (EV = 12.7) was the optimal cutoff value for CFA-based ELISA (assay accuracy = 100%). The summation of mean and 3 SDs was selected as an optimal cutoff value for both Peptide-A (EV cutoff = 2.7; assay accuracy = 100%) and Peptide-B (EV cutoff = 1.3; assay accuracy = 98%) ELISAs. Based on the optimized cutoff values, detection specificity of CFA, Peptide-A, and Peptide-B ELISAs were equally high (100%). While the CFA and Peptide-A ELISAs provided 100% detection sensitivity, the Peptide-B ELISA provided lower detection sensitivity (91%).

Table 7: Diagnostic performance of CFA- and peptide-base ELISA for detection of anti-*P. insidiosum* antibody in serum samples from 34 proven cases of human pythiosis, and 92 controls (CFA, culture filtrate antigen; ELISA, enzyme-linked immunosorbent assay; EV, ELISA value; SD, standard deviation).

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

Footnote:

[a] Mean ELISA value (EV) and standard deviation (SD) of the control sera (n=92)

[b] Sensitivity = true positive/(false negative+true positive) x 100

[c] Specificity = true negative/(false positive+true negative) x 100

[d] Accuracy (ability of an assay to provide true positive and true negative results) = (true positive+true negative)/(true positive+true negative+false positive+false negative) x 100

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

Figure 14: The UN05080-translated, 751-amino acid-long protein sequence of the putative exo-1,3-β-glucanase of *P. insidiosum*, PinsEXO1. Bold letters are 307 amino acids of the partial PinsEXO1 protein [accession number, ADI86643.1 (Krajaejun et al. 2010)] used to BLAST search the local *P. insidiosum* transcriptome database (unpublished data) to obtain an extended PinsEXO1 protein sequence. Gray boxes indicate two antigenic determinants (Peptide-A and Peptide-B, respectively) of PinsEXO1, predicted by the PREDITOP program (Pellequer and Westhof 1993).

10. สรุปและวิจารณ์ผลการทดลอง

10.1 Identification and characterization of exo-1,3-β-Glucanase gene of *P. insidiosum*

- Full length sequence ของ glucanase gene
- เปรียบเทียบ Glucanase gene sequences ของ *P. insidiosum* กับเชื้อราอื่น
- Cellular localization ของ *P. insidiosum* glucanase
- Glucanase gene expression ใน developmental stage ต่างๆ
- Glucanase function (Biochemical activity / Morphogenesis)

เมื่อไม่นานมานี้ คณะผู้วิจัยได้รายงานการพบ immunodominant protein ขนาด 74-kDalton ของเชื้อ *P. insidiosum* และจากการวิเคราะห์ทางด้าน Proteomic and genetic พบยีนที่ถอดรหัสเป็นโปรตีนนี้คือ exo-1,3-β-glucanase gene (*PinsEXO1*) (แต่ sequence ที่ได้เป็น partial sequence). Glucanase เป็นโปรตีนที่น่าสนใจ เพราะในเชื้อราบางชนิด glucanase มีบทบาทในการเปลี่ยนแปลงรูปร่างของเชื้อ, ช่วยก่อให้เกิดโรค (การเกาะติดและการชอนไชเนื้อเยื่อโฮส), และมีบทบาทในการกระตุ้นภูมิกันของร่างกาย. หน้าที่ของ *P. insidiosum* glucanas อาจจะเหมือนกับที่พบในเชื้อรา แต่ก็ยังไม่มีการศึกษา. วัตถุประสงค์ของโครงการนี้คือ เพื่อศึกษาหน้าที่ยีน *PinsEXO1* ของ *P. insidiosum* และใช้ประโยชน์จากยีนนี้ในทางคลินิก. จากการใช้เทคนิค Adaptor and RACE PCR ทำให้สามารถทราบลำดับเบสของ *PinsEXO1* ทั้งหมด และเมื่อศึกษาความสัมพันธ์ทาง phylogenetics พบว่า *PinsEXO1* มีลำดับเบสแตกต่างกับเชื้อราชนิดอื่นๆ แสดงว่าเชื้อ *P. insidiosum* มีวิวัฒนาการที่แตกต่างจากเชื้อราอื่นๆ เมื่อดูการแสดงออกของยีนพบว่า *PinsEXO1* แสดงออกสูงเมื่อเชื้อเจริญที่อุณหภูมิร่างกาย และมีการแสดงออกต่ำเมื่อเชื้อเจริญในภาวะที่ขาดคาร์บอน (เทียบกับการเจริญที่อุณหภูมิห้อง). คณะผู้วิจัยใช้วิธีทาง bioinformatics คำนาย B-cell epitopes ได้ 2 epitopes คือ Peptide-A และ -B ซึ่งมี binding activity ดีกับ anti-*P. insidiosum* antibody ใน serum ผู้ป่วย pythiosis ทั้งหมดที่ทดสอบ. จากการทำ Western blot analysis กับ crude antigens ที่สกัดได้จากเชื้อ และใช้ antibody จากกระต่ายที่สร้างต่อ synthetic Peptide-A และ B ของโปรตีน Glucanase ทำให้ทราบว่า PinsEXO1 ไม่มีการหลั่งออกมานอกเซลล์ และการใช้ immunostaining assay บ่งบอกว่าโปรตีน PinsEXO1 อยู่ที่ผนังเซลล์. ซึ่งสนับสนุนว่ายีน *PinsEXO1* น่าจะมีบทบาทต่อการเกิดโรค.

ในส่วนของการศึกษา Function ของยีน Glucanase นั้น จำเป็นต้องมี DNA transformation method (ใช้กดการแสดงออกของยีน *PinsEXO1* เพื่อดู morphology ที่อาจเปลี่ยนแปลงไป) และจำเป็นที่จะต้องมี recombinant glucanase protein (เพื่อใช้ทดสอบ biochemical activity) ซึ่ง experiments ทั้งสอง เป็นสิ่งที่คณะผู้วิจัยพยายามอย่างมากเพื่อจะทำการทดลองให้ได้ตามมุ่งหวังไว้. ในส่วนของ DNA transformation method นั้น คณะผู้วิจัยได้ทำการทดลอง 2 วิธีการ คือ (i) PEG-mdiated transformation of protoplasts (โดยประยุกต์ protocol ที่ได้มาจาก Dr. Francine Govers, Wageningen University, The

Netherlands) ซึ่งต้องอาศัยการผลิต protoplasts จำนวนมาก, และ (ii) Electroporation of zoospore (โดยประยุกต์ protocol ตามวิธีของ Weiland [48]) แต่ประสบข้อจำกัดคือ เชื้อ *P. insidiosum* สามารถใช้สร้าง protoplast และ zoospore ได้จำนวนน้อยมาก คณะผู้วิจัยได้พยายามเตรียมเชื้อจำนวนมากเท่าที่จะทำได้ (2L flask จำนวน 4 flasks) แต่ yield ของ zoospore และ protoplast ที่ได้นั้นยังน้อยมากอยู่ (น้อยกว่า 1 x 10^6 zoospores หรือ protoplasts) แต่อย่างไรก็ตาม คณะผู้วิจัยก็ได้นำ zoospores/protoplasts ที่ได้ทั้งหมดมาทดลองทั้งวิธี PEG-mdiated transformation และ Electroporation โดยใช้ Plasmid DNA จาก Dr. Howard Judelson (University of California-Davis) ซึ่งมี selection marker ต่อยา Geneticin (G418) โดยคณะผู้วิจัยพบว่าควรใช้ยา Geneticin ที่ปริมาณ 200 µg/mL ในการคัดเลือก Transfprmants. หลังจากได้พยายามและปรับปรุงวิธีการหลายครั้ง จึงพบว่าวิธี Transformation ดังกล่าวทั้งสองไม่เหมาะสมต่อ *P. insidiosum* เนื่องจากไม่ได้ Transformant ซึ่งน่าจะมีสาเหตุจากจำนวน zoospores/protoplasts น้อยเกินไป (ความถี่ของการเกิด DNA transformation ใน microorganism ใดๆ มักจะน้อยมาก). คณะผู้วิจัยค้นคว้าพบว่าการใช้วิธี DNA particle bombardment น่าจะเป็นวิธีที่เหมาะสม เนื่องจากไม่จำเป็นต้องเตรียม protoplast และ zoospore เพราะสามารถใช้สายรา hyphae ในการทำ DNA transformation ได้โดยตรง. แต่ปัญหาคือ วิธี DNA particle bombardment ต้องอาศัยเครื่องมือที่เรียกว่า Gene gun และ reagents หลายอย่างที่มีราคาสูงมาก และไม่มีอยู่ในสถาบัน. จึงเป็นอุปสรรคในการดำเนินการวิจัยขั้นต่อไปเพื่อศึกษา role ของ glucanase ต่อ morphology ของเชื้อ *P. insidiosum*.

ในส่วนของ recombinant glucanase protein เพื่อใช้ทดสอบ biochemical activity ของ glucanase นั้น คณะผู้วิจัยได้ทำ cloning and expression of *Pythium insidiosum* glucanase gene ใน bacterial system. จากการวิเคราะห์โปรตีนด้วยวิธี Western blot และใช้ anti-histidine antibody ในการ probe (Recombinant protein จะถูก Tag โดย Histidine x 6 ตัว) ทำให้พบปัญหาคือ Glucanase ที่ expressed ได้นั้นไม่ stable และเกิด degraded ทุกครั้งหลังจากที่กระตุ้นให้สร้างโปรตีนในแบคทีเรีย ถึงแม้จะเปลี่ยน Bacterial host cells หลายชนิดแล้วก็ตาม. จึงเป็นอุปสรรคในการดำเนินการวิจัยขั้นต่อไปเพื่อศึกษา biochemical activity ของ glucanase. ในส่วนของการแก้ปัญหาของการ expression ของ glucanase protein นั้น ได้ปรึกษากับ Dr. Vapiporn Phuntumart อาจารย์และนักวิจัยมหาวิทยาลัย Bowling Green State University, USA และกำลังร่วมมือกันใช้วิธี in vivo expression ใน Bacteria เพื่อใช้ทดสอบการย่อย substrate ชนิดต่างๆ บนอาหารเลี้ยงเชื้อ จึงได้ส่งนักศึกษาปริญญาเอกทุน คปก. รุ่น 11 (น.ส. อังศณา กีรติจรัส) เพื่อดำเนินการวิจัยอยู่ขณะนี้.

10.2 Clinical application of *P. insidiosum* exo-1,3-β-Glucanase gene

10.2.1 Efficiency comparison of DNA extraction methods

In the present study, conventional-extraction, salt-extraction, and rapid-extraction protocols were used to extract the gDNA of *P. insidiosum*. These protocols were characterized with regard

to efficiency and suitability of the gDNA produced for downstream applications. The high A260/A280 ratio (~1.8-2.1) of the gDNA extracted by all three methods indicates minimal protein contamination [52] (**Table 4**). DNA concentrations reported by UV absorbance measurement (NanoDrop spectrophotometer) tended to exceed estimations by fluorescence-based measurements (Qubit fluorometer) (**Table 4**), possibly because UV absorbance measures DNA and RNA together, while the fluorescence-based method specifically reports DNA concentration [52,53]. Thus, fluorometric estimation of gDNA concentration was preferred in this study.

We compared the quality and quantity of all extracted gDNA samples. The conventional-extraction protocol maximized both concentration and DNA integrity (**Table 4, Figure 9**), making this protocol more suitable for a large-scale, high quality gDNA preparation. However, the conventional-extraction protocol demanded a great deal of hyphal material (i.e., 200-1,000 mg), more extraction steps, a larger volume of reagents, and required liquid nitrogen. Alternatively, the salt-extraction and rapid-extraction protocols can be used to extract gDNA from a smaller quantities of mycelia (i.e., 50-100 mg). Unlike with conventional-extraction, these two protocols required fewer reagents and shorter extraction time (4 hr for rapid-extract; ~6-7 hr for salt-extract; 1-2 days for conventional-extract). For smaller-scale gDNA extractions, the salt-extraction protocol was preferred because it provided higher DNA concentration (114 vs 46 ng/µl), yield (145 vs 65 ng/mg), and integrity (**Figure 9**). Moreover, the salt-extraction protocol did not require phenol, chloroform, or liquid nitrogen.

To further evaluate gDNA quality, PCR amplifications targeting three different genes (*rDNA*, *EXO1*, and *UN04715*) of *P. insidiosum* were performed, using all sets of primers (**Table 5**). Amplicon sizes ranged from ~200 to ~ 1,700 bp (**Table 5**). It was observed that impaired integrity of the gDNA generated by the rapid-extraction protocol markedly limited the success of PCR amplifications, particularly with respect to long amplicons (**>** 550 bp) (**Figure 10**). The gDNA templates extracted by the conventional-extraction and salt-extraction protocols may be used to amplify an amplicon size of at least 1.7 kb (**Figure 10**), indicating that these two protocols are appropriate for PCR-based applications.

In conclusion, the conventional-extraction protocol provided the highest quality and quantity of *P. insidiosum* gDNA. This protocol was suitable for the large-scale preparation of high quality gDNA. The salt-extract was a simple, rapid, and efficient protocol, making it useful for high throughput, small-scale preparation of gDNA for many molecular-biological experiments and applications. Although the rapid-extract protocol had the shortest turnaround time, the gDNA

obtained was of limited quality and quantity. These observations may instruct investigators and aid their determination of which extraction protocol is optimal for a given application, dependent upon quality and quantity of gDNA desired, availability of materials and equipment, and allowable turnaround time.

10.2.2 Development of molecular-based diagnostic assay

Molecular diagnostic techniques for pythiosis are useful if microbiological and immunological assays are not available, or there is a suspected case that tested negative by other methods. So far, PCR identification of *P. insidiosum* has been largely relied on amplification of the rDNA region [54]. As an alternative assay, we evaluated diagnostic performance of the PCR primers, Dx3 and Dx4, which target *PinsEXO1* of *P. insidiosum* [49], in comparison with the previously-reported rDNA-specific primers, ITSpy1 and ITSpy2 [38]. The primer Dx3 and Dx4 were designed by using the well-established program called Primer-BLAST [55], which is publicly available at the NCBI website (http://www.ncbi.nlm.nih.gov/tools/primer-blast). Based on a given target DNA sequence (i.e., *PinsEXO1* gene), the Primer-BLAST program selects the most suitable primer set and uses the BLAST and global alignment algorithm to evaluate the selected primers for any cross annealing against all genes deposited in the NCBI nucleotide database. Thus, the resulting primers (Dx3 and Dx4) should be specific to the target sequence.

The PCR template was adequately present in all reactions, because the ITS1/4 universal primers can amplify an expected amplicon from all 83 gDNA samples included in this study. The no-template control (included in each round of PCR assays) was all read negative, indicating that there was no DNA contamination that could lead to a false positive result. To evaluate detection specificity, we collected 48 different culture-proven fungal microorganisms (**Table 1**), as the controls in the PCR assay evaluation. We also included a number of *Aspergillus* spp. and *Zygomycetes*, because these fungi have microscopic features that could be confused with *P. insidiosum*. When the Dx3/4 and ITSpy1/2 primers were used to amplify gDNA prepared from the fungal controls (**Table 1**), no PCR product was observed by gel electrophoresis. This result indicated that there was no non-specific primer annealing to gDNA of the fungal controls, and thus, both primer sets equivalently had 100% detection specificity.

To evaluate detection sensitivity, gDNA prepared from all 35 culture-proven *P. insidiosum* isolates were PCR amplified by using the Dx3/4 and ITSpy1/2 primers. The ITSpy1/2 primers can amplify an expected amplicon from 32 out of 35 isolates tested, although three isolates (P11,

NAN06, and SIMI7695.48) of which could be read negative due to a very faint PCR band (**Figure 12B**). In addition, gDNA of a few *P. insidiosum* isolates (P1, P22, and Pi-S) gave an unexpected smaller amplicon (~220 bp rather than ~230 bp), suggesting some variations in the rDNA region of *P. insidiosum* (**Figure 12B)**. Altogether, the detection sensitivity of the ITSpy1/2 primers was compromised, and thus, calculated to be 91%. On the other hand, based on the same set of gDNA samples, the Dx3/4 primers successfully amplified an expected intense PCR product (~550 bp) from all *P. insidiosum* gDNA samples tested (**Figure 12A)**. Therefore, the Dx3/4 primers had a better detection sensitivity (100%), when compared to that of the ITSpy1/2 primers (91%).

In conclusion, we demonstrated here that, when amplifying the gDNA templates of 35 *P. insidiosum* isolates and 48 fungal controls (**Table 1**), the ITSpy1/2 primers had excellent detection specificity (100%), but limited detection sensitivity (91%). By using the same set of templates for PCR amplification with the Dx3/4 primers, it was found that *PinsEXO1* was a more efficient PCR target (100% detection specificity and sensitivity) and it can be an alternative marker for molecular identification of *P. insidiosum*.

10.2.3 Efficiency comparison of serodiagnostic assays

We used CFA to develop an in-house ELISA for detection of anti-*P. insidiosum* antibody. We transformed each ELISA OD signal to EV in order to minimize the OD fluctuations between batch tests. Calculation of EV was helpful for monitoring anti-*P. insidiosum* antibody levels in patients before and after treatment [32]. By using the mean+4SDs cutoff (EV=2.1), the assay provided 100% detection sensitivity and specificity. The EV cutoff selected for this CFA-based ELISA is comparable to the cutoff (EV=2.0) used in the previously-reported SABH-based ELISA [32]. This new ELISA exhibits excellent reproducibility as the EVs of positive control serum from different batches were almost identical (CV = 1.4%).

Serological assays including ID, WB, ELISA, ICT and HA have been developed for diagnosis of pythiosis [19,31–34,38,56–61]. Which assay is most efficient and suitable for clinical use needs to be determined. We compared performances of ID, ICT, HA and the newly developed ELISA. WB was excluded from the comparison because its procedure is complex and it requires equipments not available in routine clinical laboratories. In this study, CFA was used to develop all assays. Use of serodiagnostic tests for diagnosis of ocular pythiosis is not recommended because infection of immune privilege site like ocular tissue poorly induces antibody production [33,34]. HA developed here showed the lowest diagnostic performance (**Table 6**). Jindayok et al.

previously reported HA with a better detection sensitivity (88%) and specificity (99%) [34]. Diagnostic performance of HA might be impacted by the quality of sheep red blood cells used, and cross-reactive sera from patients exposed to sheep products. Regarding cross reactivity of CFA against other antibodies, the 100% detection specificity reported for ELISA, ICT and ID (**Table 6**) indicated that CFA used to prepared these assays showed no cross-reactivity to antibodies in the control sera, including sera from patients with other fungal infections, as previously described [31,33,57,59].

Procedures, result interpretations and turnaround time of each assay were also compared (**Table 6**). ID, ICT and HA are simple, one-step procedures, while ELISA requires specialized equipment (i.e., ELISA plate reader and incubator) and multiple steps. ELISA results were unambiguously interpreted based on EVs determined by the ELISA plate reader. Results of ID, ICT and HA must be subjectively determined by visual estimation, leaving some potential for error depending upon the experience/inexperience of laboratory personnel. ICT has the shortest turnaround time from application of serum sample to reading a result (~30 min). HA and ELISA required a few hours to finish all assay procedures, while the ID assay had the longest turnaround time, requiring an overnight incubation.

In conclusion, ICT and CFA-based ELISA had the highest diagnostic performance. Determination of which serological test is suitable for a given diagnostic laboratory depends on the availability of materials and equipment, the experience of laboratory personal, the diagnostic performance desired and allowable turnaround time. Understanding the advantages and disadvantages associated with each assay can aid in both appropriate assay selection and the interpretation of test results, which, it is hoped, will lead to more rapid diagnosis and improved healthcare for patients with pythiosis.

10.2.4 Development of serodiagnostic assay

PREDITOP is a useful program for identifying peptides with potential epitopes in a protein of interest [50]. In fact, an epitope is capable of inducing antibody production, and in turn, the antibody produced would bind to the corresponding epitope. The PREDITOP program is based on “turn prediction”, and a peptide chosen is invariably very hydrophilic [50]. Thus, the peptide is likely to appear on protein surface, and could lead to robust antibody production or binding. Two antigenic epitopes (Peptide-A and Peptide-B) were predicted in the UN05080-translated protein (**Figure 14**). The Peptide-A and the Peptide-B were tested for their recognitions by anti-*P.*

insidiosum antibodies in sera from patients with pythiosis. ELISA was selected as the assay platform because of its superior diagnostic performance over the other assays [51]. Additionally, while a result of other assays is subjectively determined by visual estimation, an ELISA result is unambiguously determined by a machine, which can prevent some interpretation error by laboratory personnel [51].

Because a peptide is a short stretch of amino acids, it could be poorly coated onto an ELISA plate, or it could adhere to plastic surface in a way that masks its antigenic epitope. This could lead to limited interaction of the peptide and its corresponding antibody. To solve this obstacle, each of Peptide-A and Peptide-B was conjugated to biotin. The biotin is efficiently and specifically captured by avidin, a molecule pre-linked on an ELISA plate. This strategy could enhance an efficiency of ELISA plate coating, and therefore, promote peptide-antibody interaction. Peptide-A and Peptide-B ELISAs were evaluated against a total of 34 pythiosis and 92 control sera. Both assays can discriminate EVs of the pythiosis sera from EVs of the control sera. However, overall diagnostic performance of Peptide-A ELISA (100% sensitivity, specificity, and accuracy) was better than that of Peptide-B ELISA (91% sensitivity, 100% specificity, and 98% accuracy). Although the epitope prediction program (i.e., PREDITOP) could increase chance to successfully obtain a peptide candidate with an antigenic epitope, it cannot guarantee that all predicted epitopes (i.e., Peptide-A and Peptide-B) are efficient for antibody production or binding. Thus, extensive evaluation of more than one peptide is crucial and necessary. As seen here, Peptide-A is a more efficient epitope than Peptide-B. We have collected the pythiosis sera as many as possible for the assay evaluation. However, because the disease is relatively rare, the number of sera from pythiosis patients was limited (n=34). Further evaluation using more pythiosis sera will be useful to more accurately address the detection sensitivity of the assays.

The CFA-based ELISA has been recently developed and becoming a reference assay for serodiagnosis of pythiosis [51]. Diagnostic performances of the Peptide-A ELISA and the CFA-based ELISA were compared. It was notable that the mean EVs of the pythiosis sera determined by the CFA-based ELISA (EV = 145.9) was 19-fold higher than that by the Peptide-A ELISA (EV = 7.7). However, there was slightly different (2.4 folds) for the mean EVs of the control sera determined by both assays. This may be due to the presence of hundreds of different antigenic epitopes in the CFA protein mixture, compared to only one epitope in the Peptide-A. Nevertheless, both assays provided equivalently-high diagnostic performance (100% sensitivity, specificity, and accuracy; **Table 7**).

In conclusion, we report here the use of synthetic PinsEXO1 peptides for serodiagnosis of human pythiosis. The detection specificity of CFA-based, Peptide-A, and Peptide-B ELISAs were equally high (100%). While Peptide-B ELISA had limited detection sensitivity (91%) and accuracy (98%), both Peptide-A and CFA-based ELISAs showed equivalently high detection sensitivity (100%) and accuracy (100%). Thus, the synthetic Peptide-A can be used as an alternative and efficient antigen for development of a serodiagnostic test for pythiosis. Synthetic peptide could address the concern on the batch-to-batch variation, as well as, significant degradation of the crude protein extract. Since peptides are non-infectious and commercially-available, they can be safely handled and prepared for use in a routine diagnostic laboratory.

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12. Output จากโครงการวิจัยที่ได้รับทุนจาก สกว.

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3. Keeratijarut A, Lohnoo T, Yingyong W, Nampoon U, Lerksuthirat T, Onpaew P, Chongtrakool P, **Krajaejun T***. PCR Amplification of a Putative Exo-1,3-β Glucanase Gene for Identification of the Pathogenic Oomycete *Pythium insidiosum* (**Revision**)
4. Lohnoo T, Jongruja N, Rujirawat T, Yingyong W, Lerksuthirat T, Nampoon U, Kumsang Y, Onpaew P, Chongtrakool P, Keeratijarut A, Brandhorst TT, **Krajaejun T***. Efficiency Comparison of Three Methods for Extracting Genomic DNA of the Pathogenic Oomycete *Pythium insidiosum* (**Submitted**)
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6. Keeratijarut A, Lohnoo T, Yingyong W, Nampoon U, **Krajaejun T***. Immunological and Genetic Characterization of a Putative Exo-1,3-β Glucanase Gene from the Pathogenic Oomycete *Pythium insidiosum* (**In preparation**)

12.2 การนำผลงานวิจัยไปใช้ประโยชน์

- เชิงวิชาการ: โครงการวิจัยนี้เป็นส่วนหนึ่งของการสร้างนักวิจัย 2 ท่าน คือ *ระดับปริญญาเอก 1 ท่าน* ในหลักสูตร Molecular medicine คือ น.ส. อังศณา กีรติจรัส (ทุน คปก. รุ่นที่ 11) ซึ่งคาดว่าจะสำเร็จการศึกษาในปีการศึกษา 2556/7 มีงานวิจัย 3 เรื่อง คือ

- Peptide ELISA for Antibody to *Pythium insidiosum* Based on Predicted Antigenic Determinants of Exo-1,3-β-Glucanase
- PCR Amplification of a Putative Exo-1,3-β Glucanase Gene for Identification of the Pathogenic Oomycete *Pythium insidiosum*
- Immunological and Genetic Characterization of a Putative Exo-1,3-β Glucanase Gene from the Pathogenic Oomycete *Pythium insidiosum*

ระดับหลังปริญญา 1 ท่าน ในหลักสูตรฝึกอบรมแพทย์ประจำบ้าน สาขาวิชาพยาธิวิทยาคลินิก คือ น.พ. ถกล เจริญศิริสุทธิกุล ซึ่งจะสำเร็จการศึกษาในปีการศึกษา 2556/7 โดยมีผลงานตีพิมพ์ 1 เรื่อง คือ Chareonsirisuthigul et al., Diagn. Microbiol. Infect. Dis. 2013;76(1):42–5.

12.3 <u>อื่น ๆ</u>

- หนังสือ

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- การจดสิทธิบัตร:

1. Pongernnak P, Sriwanichrak K, Phasomsap A, Krajaejun T; Mahidol University, assignee. [Molecular Markers for Size Estimation of DNA]. (Submitted)

- การเสนอผลงานในที่ประชุมวิชาการ

1. Oral presentation: The Oomycete Molecular Genetics Research Collaboration Network meeting, Nanjing, China (2012). Title: Whole Genome Gene Discovery in *Pythium insidiosum* (การค้นหาและวิเคราะห์ glucanase gene family เป็นส่วนหนึ่งของงานวิจัย)
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Mycology

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

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ABSTRACT

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

1. Introduction

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

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

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

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

2. Materials and methods

2.1. Serum samples

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

2.2. Antigen preparation

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

2.3. ELISA

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

2.4. ID

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

2.5. ICT

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

2.6. HA

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

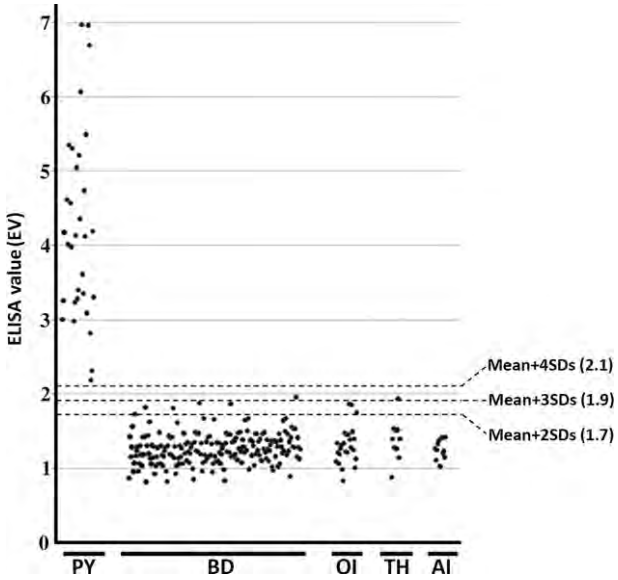

Fig. 1. Detection of anti-*P. insidiosum* antibody in sera by ELISA. Three ELISA cutoffs were calculated from the mean value (EV = 1.28) of the control sera (BD, OI, TH, and AI) plus 2, 3 and 4 SDs (1 SD = 0.21). Five EVs (range, 8.4–14.6) of the PY serum samples were not shown in the plot. Abbreviations: EV, ELISA value; PY, patients with pythiosis (n = 37); BD, healthy blood donors (n = 200); OI, patients with other infectious diseases (n = 25); TH, healthy thalassemic patients (n = 10); AI, patients with autoimmune diseases (n = 13).

agglutination was read as a positive test result, whereas the absence of agglutination was read as a negative test result.

2.7. Statistical analysis

Sensitivity, specificity, positive predictive value (PPV), negative predictive values (NPV), accuracy, mean value of EV, SD, EV-cutoff point and CV were calculated using the Microsoft EXCEL2007 program.

3. Results

3.1. Development of CFA-based ELISA

Mean EVs of pythiosis (n = 37) and control (n = 248) sera were 5.1 (range, 2.2–14.6; SD, 2.7) and 1.3 (range, 0.8–2.0; SD, 0.2), respectively. Three cutoff points (EV = 1.7, 1.9, and 2.1) were calculated based on summation of the mean EV of control sera and 2 (mean + 2SDs), 3 (mean + 3SDs) and 4 (mean + 4SDs) SDs, respectively (Fig. 1). The mean+2SDs, +3SDs, and +4SDs cutoffs provided 96%, 99%, and 100% detection specificity, respectively. All the cutoffs provided 100% detection sensitivity. The mean+4SDs EV was selected as the cutoff of ELISA because all pythiosis sera were determined to be positive and all control sera were determined to be negative. EVs of the pooled positive serum from seven independent batch tests provided mean value of 8.6, SD of 0.1 and CV of 1.4%.

3.2. Diagnostic performances of ELISA, ID, ICT, and HA

ELISA, ID, ICT, and HA were compared for performances in detection of anti-*P. insidiosum* antibody using the same set of serum samples. All assays, except HA, were tested against 37 pythiosis and 248 control sera. HA was evaluated with fewer sera (32 pythiosis and 208 control sera) due to unavailability of some samples. To serve as the system controls and ensure accurate result interpretation, all assays were also tested with the same pooled positive and negative sera. For an ID test to be considered positive, 1–3 precipitins of the pooled positive serum formed line(s) of identity with precipitins of a test serum. Detection sensitivity of ELISA and ICT (100%) were markedly higher than ID (68%) and HA (84%) (Table 1). Detection specificity of all serological assays were equivalently high (100%), except HA (82%) (Table 1). Accuracies (ability of an assay to provide true positive and true negative results) of ID, ELISA, ICT and HA were 96%, 100%, 100%, and 83%, respectively. PPVs of ID, ELISA and ICT were 100%, while PPV of HA was relatively low (42%). All assays showed high NPVs (range, 95–100%; Table 1).

4. Discussion

We used CFA to develop an in-house ELISA for detection of anti-*P. insidiosum* antibody. We transformed each ELISA OD signal to EV in order to minimize the OD fluctuations between batch tests. Calculation of EV was helpful for monitoring anti-*P. insidiosum* antibody levels in patients before and after treatment (Krajaejun et al., 2002). By using the mean+4SDs cutoff (EV = 2.1), the assay provided 100% detection sensitivity and specificity. The EV cutoff selected for this CFA-based ELISA is comparable to the cutoff (EV = 2.0) used in the previously-reported SABH-based ELISA (Krajaejun et al., 2002). This new ELISA exhibits excellent reproducibility as the EVs of positive control serum from different batches were almost identical (CV = 1.4%).

Serological assays including ID, WB, ELISA, ICT and HA have been developed for diagnosis of pythiosis (Mendoza et al., 1986, 1992, 1997; Imwidthaya and Srimuang, 1989; Pracharktam et al., 1991; Grooters et al., 2002; Krajaejun et al., 2002, 2006a, 2009; Vanittanakom et al., 2004; Supabandhu et al., 2009; Jindayok et al., 2009). Which assay is most efficient and suitable for clinical use needs to be determined. We compared performances of ID, ICT, HA, and the newly developed ELISA. WB was excluded from the comparison because its procedure is complex and it requires equipments not available in routine clinical laboratories. In this study, CFA was used to develop all assays. Use of serodiagnostic tests for diagnosis of ocular pythiosis is not recommended because infection of immune privilege site like ocular tissue poorly induces antibody production (Jindayok et al., 2009; Krajaejun et al., 2009). HA developed here showed the lowest diagnostic performance (Table 1). Jindayok et al., previously reported

Table 1
Performance of ID, ELISA, ICT and HA in the detection of anti-*P. insidiosum* antibody in serum samples.

Assay[a]	Sensitivity[b] (%)	Specificity[c] (%)	Accuracy[d] (%)	PPV[e] (%)	NPV[f] (%)	Assay procedure	Interpretation of test result[g]	Turnaround time (h)
ID	68	100	96	100	95	Single-step	Subjective	24
ELISA	100	100	100	100	100	Multi-step	Objective	3
ICT	100	100	100	100	100	Single-step	Subjective	0.5
HA	84	82	83	42	97	Single-step	Subjective	1

[a] ID, ELISA, and ICT were tested with all 37 pythiosis and 248 control sera. Due to limited sample availability, HA was evaluated with 32 pythiosis and 208 control sera.
[b] Sensitivity = true positive/(false negative + true positive) × 100.
[c] Specificity = true negative/(false positive + true negative) × 100.
[d] Accuracy = (true positive + true negative)/(true positive + true negative + false positive + false negative) × 100.
[e] PPV = true positive/(false positive + true positive) × 100.
[f] NPV = true negative/(false negative + true negative) × 100.
[g] Determination of result via visual assessment is referred to as "subjective" interpretation. Quantification via instrument is referred to as "objective" interpretation.

HA with a better detection sensitivity (88%) and specificity (99%) (Jindayok et al., 2009). Diagnostic performance of HA might be impacted by the quality of sheep red blood cells used, and cross-reactive sera from patients exposed to sheep products. Regarding cross reactivity of CFA against other antibodies, the 100% detection specificity reported for ELISA, ICT, and ID (Table 1) indicated that CFA used to prepared these assays showed no cross-reactivity to antibodies in the control sera, including sera from patients with other fungal infections, as previously described (Mendoza et al., 1986; Imwidthaya and Srimuang, 1989; Pracharktam et al., 1991; Krajaejun et al., 2009).

Procedures, result interpretations and turnaround time of each assay were also compared (Table 1). ID, ICT, and HA are simple, one-step procedures, while ELISA requires specialized equipment (i.e., ELISA plate reader and incubator) and multiple steps. ELISA results were unambiguously interpreted based on EVs determined by the ELISA plate reader. Results of ID, ICT, and HA must be subjectively determined by visual estimation, leaving some potential for error depending upon the experience/inexperience of laboratory personnel. ICT has the shortest turnaround time from application of serum sample to reading a result (~30 min). HA and ELISA required a few hours to finish all assay procedures, while the ID assay had the longest turnaround time, requiring an overnight incubation.

In conclusion, ICT and CFA-based ELISA had the highest diagnostic performance. Determination of which serological test is suitable for a given diagnostic laboratory depends on the availability of materials and equipment, the experience of laboratory personal, the diagnostic performance desired and allowable turnaround time. Understanding the advantages and disadvantages associated with each assay can aid in both appropriate assay selection and the interpretation of test results, which, it is hoped, will lead to more rapid diagnosis and improved healthcare for patients with pythiosis.

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96 *Pythium*

Theerapong Krajaejun, Boonmee Sathapatayavongs, and Thomas D. Sullivan

CONTENTS

96.1 INTRODUCTION

Pythiosis is a life-threatening infectious disease caused by the fungus-like organism, *Pythium insidiosum*, a *Pythium* species that was first introduced and validly published by De Cock et al. in 1987.[1,2] In fact, the disease first occurred in horses in the middle of the nineteenth century, and the causative agent was first isolated as an unidentified nonsporulated filamentous fungus in 1901 by two Dutch researchers, de Haan and Hoogkamer.[1,2] In 1974, Austwick and Copland reported that the pathogen isolated from horses should be placed in the genus *Pythium* because it produces biflagellate zoospores, which represent a distinctive morphological phase of members of this genus.[3] *P. insidiosum* has been recognized by other names, including *Hyphomyces destruens*, *Pythium gracile*, and *Pythium destruen*; and the disease pythiosis has also been known under several different names, including hyphomycosis destruens, phycomycosis, swamp cancer, bursattee, kunker, cutaneous habronemiasis, granular dermatitis, espundia, and leeched.[1–4] In addition to horses, pythiosis has been increasingly reported in other animals, including dog, cat, cattle, bear, and sheep.[2] The first patient with human pythiosis was found in New Zealand in 1984, but the case was not reported in the literature until 1997.[5] In 1985, two human pythiosis cases were diagnosed in Thailand.[6] Since then, human pythiosis has been documented from other countries: United States, Haiti, Brazil, Malaysia, and Australia.[7–11]

The hyphal phase of *P. insidiosum* is hard to distinguish from true filamentous fungi, such as *Aspergillus* spp., *Penicillium* spp., and *Zygomycetes*, which cause infections with similar clinical features to pythiosis.[12] This can lead to the misdiagnosis of patients with pythiosis. For this and other reasons, morphological characterization and culture identification of *P. insidiosum* are difficult and time-consuming, and therefore, diagnosis of pythiosis is often delayed. Conventional antifungal agents are not effective for treatment of *P. insidiosum* infection because *Pythium* spp. lack the drug-targeted ergosterol biosynthesis pathway.[13] Because β-glucan is a major cell wall component of fungi and *Pythium* species,[14] Pereira recently tested the in vivo effect of caspofungin (a new antifungal drug that inhibits β-glucan synthase) on *P. insdiosum* infection in a rabbit model of pythiosis.[15] While the drug slowed the growth of lesions, it was unable to eliminate the pathogen in the infected animals.[15] Without a useful chemotherapeutic agent, the main treatment approach for pythiosis is radical surgery, which should be urgently performed to limit the progression of the disease. Many patients die as a result of advanced and aggressive infection. Early and correct diagnosis of pythiosis is crucial for proper treatment and a better prognosis.

Pythiosis should be regarded as an emerging infectious disease. Many health care professionals are not familiar with this disease or its causative agent, *P. insidiosum*. The increasing diagnosis of pythiosis from all over the world indicates that more clinicians and microbiologists are aware of pythiosis and that the disease may not be uncommon. Our limits of knowledge regarding pythiosis and the lack of diagnostic and treatment options bring about a high morbidity and mortality rate for pythiosis patients. In this chapter, up-to-date information about pythiosis and *P. insidiosum* are described in detail, with a focus on diagnostic modalities.

96.1.1 Classification, Morphology, and Biology

P. insidiosum is recognized as a member of the oomycetes,[14,16] which is a unique group of eukaryotic microorganisms with a fungal-like morphology that are physiologically, biochemically, and phylogenetically different from other eukaryotic pathogens, that is, fungi and parasites, and are most closely related to the brown algae.[14] Taxonomically, *P. insidiosum* was previously classified in the family Pythiaceae, order Pythiales, class Oomycetes, and the phylum Pseudofungi in the kingdom Chromista.[2] According to the recently published phylogenetic tree of eukaryotes, *P. insidiosum* belongs to the oomycetes within the Stramenopiles of the supergroup Chromalveolates.[17] Genera within the oomycetes include *Pythium*, *Albugo*, *Peronospora*, *Phytophthora*, *Plasmopara*, *Bremia*, *Aphanomyces*, *Lagenidium*, and *Saprolegnia*.[16] Although the majority of the oomycetes are saprophytes, many of them are pathogenic on a broad range of hosts, including plants and animals.[16] Plant pathogenic oomycetes cause significant economical loss of agricultural products each year, and the animal pathogens among the oomycetes (e.g., *Pythium* spp., *Aphanomyces* spp., *Lagenidium* spp., and *Saprolegnia* spp.) can cause devastating diseases.[16]

P. insidiosum is the only known oomycete capable of infecting both humans and other animals and the resulting disease (pythiosis) can be fatal.[2,16] In nature, *P. insidiosum* inhabits swampy areas and commonly presents in two forms: mycelia and asexual zoospores.[18] *P. insidiosum* has broad (4–10 μm), right-angle branching and sparsely septate hyphae (Figure 96.1), which are indistinguishable from the true filamentous fungi.[1,2] The zoospore, a specific character of *P. insidiosum* and other oomycetes, is produced through zoosporogenesis, which is initiated by the development of a zoosporangium (zoospore sac) at the hyphal tip and results in the formation of 5–60 zoospores within the sac.[18–20] The whole process of zoosporogenesis (generation, maturation, and release of zoospores) takes ~35 min to finish.[18] Zoospores have two motile flagella, which facilitate movement in water for 10–15 min before sequential events of detachment of flagella, encystment, hyphal germination, and colonization of one of several water plants.[18] Unlike other *Pythium* species, *P. insidiosum* occasionally produces oogonia, which are smooth and subglobose.[1,2] During mating, several antheridia, from different hyphae, attach to a single oogonium, and the tip of each antheridium produces a fertilization tube that penetrates the oogonial wall for fertilization.[1,2]

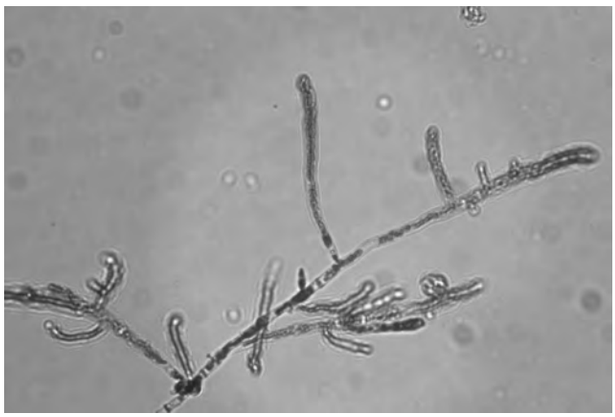

FIGURE 96.1 *P. insidiosum* observed under a light microscope.

Pythiosis has been increasingly reported mostly from tropical and subtropical countries, and also from temperate areas.[2,21] Pythiosis in animals is found worldwide, but the disease in humans has been diagnosed almost exclusively in Thailand.[6,22–31] Patients with human pythiosis in Thailand are found nationwide, indicating that the causative agent, *P. insidiosum*, is widely distributed throughout the country.[6] Because of similar ecology and climate, *P. insdiosum* is expected to inhabit neighboring countries, and unreported pythiosis cases may exist throughout Southeast Asia. A case of human pythiosis reported from Malaysia supports this speculation.[7] A recent study showed that the majority of pythiosis patients (86%) are in the working-age population (20–60 years old), are male (71%), and have agricultural-related careers, including farming, domestic husbandry, and fishing (75%).[6]

The ecological niche of *P. insidiosum* has been described by two groups, Miller[32] and Supabandhu et al.[33] Miller has successfully isolated the organism in water samples from different locations in Queensland, Australia, using a human hair-baiting method.[32] Supabandhu et al. successfully used the same method to isolate the organism in agricultural areas of northern Thailand.[33] These are important findings, which demonstrate *P. insidiosum* in aquatic environments and suggest that patients may acquire *Pythium* infection from exposure to the swampy habitats of the pathogen.

The phylogenetic relationships of *P. insidiosum* isolated from patients and animals living in different geographic areas was first reported by Schurko et al.[34,35] This group genetically classified *P. insidiosum* based on the analysis of rDNA sequences from 17 animal isolates and 6 human isolates from around the world. They demonstrated that there are three phylogenetic groups associated with the geographic origin of the organism: clade-I containing only western hemisphere isolates, clade-II containing American, Asian, and Australian isolates, and clade-III containing two American isolates and two Thai isolates. In a later study, Supabandhu et al. reported a phylogenetic analysis of 4 human isolates and

59 environmental isolates from water collected at different agricultural sites in Thailand.[33] They showed that 3 human isolates and 41 environmental isolates (70% of all isolates) are grouped in clade-II, and the remaining 1 human isolate and 18 environmental isolates (30%) belonged in clade-III. In neither of the two studies were clade-I isolates reported from Thailand.

96.1.2 Clinical Features

96.1.2.1 Human Pythiosis

96.1.2.1.1 Cutaneous/Subcutaneous Pythiosis

Patients present with an infection of cutaneous/subcutaneous tissue, characterized by chronic swelling and a painful subcutaneous, granulomatous infiltrative lump and ulcer on the face, arm, or leg. Histological examination of infected cutaneous/subcutaneous tissue reveals chronic infection with eosinophilia.[6,22] Patients with cutaneous/subcutaneous pythiosis are reported as having a good response to saturated solution of potassium iodide (SSKI) along with surgical debridement.[6] Acute infection can occur, as evidenced in a Thai patient with acute cellulitis in both legs.[31] An isolated case of cutaneous/subcutaneous infection from the United States was successfully treated with a combination of itraconazole and terbinafine.[9]

96.1.2.1.2 Vascular Pythiosis

Arterial insufficiency syndrome of lower extremities is the main feature of vascular pythiosis.[6] The syndrome ranges from intermittent claudication or resting pain to gangrenous ulceration. The infection can occur in one or both legs. Patients delay seeking medical attention on average of 91 days (range, 7–365 days) after the first observation of symptoms.[6] Angiographic findings usually demonstrate occlusion or aneurysm of arteries of the lower extremities or the aorta. On histological examination, *P. insidiosum* resides in the arterial wall or in obstructive thrombi in the arterial lumen. *Pythium* infection is not detected in venous tissue. In advanced cases, a major cause of death is from rupture of an aortic aneurysm.

Conventional antifungal agents and SSKI have had no favorable responses in vascular pythiosis. Radical surgery is the main treatment of choice. The infected tissue is removed by resection of the infected artery or limb amputation, depending on the level and severity of the infection. Most of the treated cases (~80%) of vascular pythiosis underwent amputation. Forty percent of patients with vascular pythiosis die from the infection, while 60% are cured, but left with physical handicaps. Reoccurrence is possible if surgery fails to completely remove the infection.

Recently, an immunotherapeutic vaccine prepared from crude extract of *P. insidiosum* has been used for treatment in humans[6,27,30] and animals[36–39] with pythiosis. The vaccine had favorable responses in 60% of horses, 97% of cattle, and 33% of dogs with pythiosis.[39] In humans, the *P. insidiosum* vaccine was used as the last resort in 12 patients with evidence of inadequate surgery.[6,27,30] Final outcomes of the vaccine immunotherapy were available for nine patients, of which five were cured (56%), while two died (22%), and two had persistent infections (22%).

96.1.2.1.3 Ocular Pythiosis

Patients usually present with corneal ulcer or keratitis. Other signs and symptoms include pain, irritation, decreasing visual acuity, eye lid swelling, conjunctival injection, corneal infiltrates, perforated cornea, and hypopyon. Endophthalmitis can occur in advanced cases. No cases of the infection spreading beyond the eye have been reported. Ocular pythiosis patients come to the hospital within a few days or weeks after they first notice symptoms. History of ocular trauma or corneal abrasion is common. Hyphae of *P. insidiosum* in KOH preparation of corneal scraps or discharges can be demonstrated by a simple microscopic examination. Histopathology usually reveals *P. insidiosum* located in the corneal stroma.

Like vascular pythiosis, the main treatment option for ocular pythiosis is radical surgery to get rid of all infected tissue. Eye removal by enucleation or evisceration is performed after keratectomy or keratoplasty fails to control the infection. In the minority of cases (20%), the eye is saved after anterior lamella or penetrating keratoplasty, while the majority of patients (~80%) eventually underwent enucleation or evisceration. No fatal outcomes have been reported in ocular pythiosis patients.

96.1.2.1.4 Unusual Form of Pythiosis

Some pythiosis cases with unusual site of infection, such as, the gastrointestinal tract, brain, or rhinosinus, have been reported.[6] It should be noted that gastrointestinal tract infection is a common form in animal pythiosis and may result from drinking *P. insidiosum*-contaminated water.[2] For the infections of brain tissue and rhinosinus, the pathogen may invade directly to these sites through the nasal cavity. Even with medical attention, all these cases have been fatal.

96.1.2.2 Animal Pythiosis

Pythiosis in animals is more commonly reported than the disease found in humans. Various animals are susceptible to an infection caused by *P. insidiosum*.[2] Among them, horses and dogs are the two most frequently affected hosts.[40–54] Pythiosis in some other animals, that is, calve, cat, sheep, camel, tiger, jaguar, and polar bear, has also been reported but is less common.[55–62] Animals with pythiosis present with a broad range of clinical features and with an invariably high rate of morbidity and mortality. In contrast to human pythiosis, for which vascular and ocular infections are the most common, animal pythiosis most commonly features cutaneous/subcutaneous infection of a limb (most common site), chest, abdomen, face, genitalia, tail or gastrointestinal tract infection of the pharynx, esophagus, stomach, or intestines. Other internal organs, that is, lung, bone, liver, pancreas, uterus, and internal lymph nodes can also be affected in invasive or disseminated pythiosis cases.

Animals with cutaneous/subcutaneous pythiosis mainly present with ulcerating lesions.[2] In more advanced cases, ulcers with multiple draining tracts and serosanguineous discharge can be observed. *P. insidiosum*-infected lesions can look like a tumor, with a diameter up to 50 cm (this could be the origin of the name "swamp cancer"). Animals with gastrointestinal pythiosis usually present with dysphagia, vomiting, anorexia, weight loss, diarrhea, abdominal mass, mucosal ulceration, and wall thickening of the stomach and intestines. Histological examination of infected lesions reveals granulomatous tissue with infiltration of eosinophils, neutrophils, macrophages, and giant cells. Splendore-Hoeppli-like phenomenon, including radiating eosinophilic deposits of host cell debris and pathogen antigens surrounding the fungus, is often microscopically observed in infected tissues. At the infected site, an eosinophilic coral-like mass (diameter up to 9 cm) called "kunker," which contains *P. insidiosum* hyphae, is a characteristic of pythiosis found in animals.[2] The kunker has never been described in human tissue infected with *P. insidiosum*. Like human patients, radical surgery is the main treatment option. Incomplete removal of infected tissues leads to recurrent *P. insidiosum* infection. Immunotherapy using *P. insidiosum* antigen extract has had limited efficacy although there are reports of favorable response to this treatment option.[2]

96.1.3 Pathogenesis

In nature, *P. insidiosum* completes its life cycle in aquatic environments.[2,18,32] The life cycle starts with hyphal colonization on plants and produces a zoosporangium with multiple, asexual biflagellate zoospores. Released zoospores swim and adhere to neighboring plants for colonization, and a new loop of this usual life cycle begins. Miller[32] and Mendoza et al.[18] showed that the swimming zoospores of *P. insidiosum* are strongly attracted to plants and animal and human hair, possibly by chemotaxis. The zoospores are observed to accumulate at the follicular end of the hair shafts. The zoospore is thought to be the form that initiates infection and, after conversion to the hyphal form, causes the immune responses and pathology in the host. This belief is based on the successful reproduction of pythiosis in an experimental rabbit model using zoospore inoculation.[63] However, experimental pythiosis can also be reproduced in the rabbit model by either hyphal or kunker inoculation.[63] Upon attachment of zoospore to host tissue, the flagella detach and surface amorphous substances are generated, which may facilitate adhesion to the host.[2,18] Mendoza et al. report that a zoospore tends to adhere to a cut edge of skin rather than to an intact surface.[18] Ravishankar et al. studied hyphal tip exertion forces of *P. insidiosum* and demonstrated that the pathogen is unable to penetrate undamaged cutaneous tissue.[64] They propose that the organism requires secreted proteinases to reduce tissue strength, prior to successful tissue penetration.

Direct contact of host surfaces (i.e., skin, eye) to *P. insidiosum* in its aquatic habitats is the likely route of entry for cutaneous/subcutaneous and ocular infections. Occurrence of arterial infection is thought to be a result of direct extension of *Pythium* infection from the skin.[27] The infection progresses from distal to proximal in an infected artery and causes inflammation, fibrosis, and aneurysm. Many cases of gastrointestinal pythiosis have been reported, and for these, the site of entry is likely direct contact of *P. insidiosum* with the gastric or intestinal wall, as a consequence of drinking water contaminated with the pathogen. In animals, the spread of the pathogen to other internal organs (e.g., liver, spleen, bone) may occur via the vascular system or by lymphatic drainage.[2,43,63] No information on transmission of pythiosis between animals and humans has been reported.

A striking comorbidity exists for human patients with vascular, cutaneous, and the unusual forms of pythiosis: all have an underlying hematological disorder, 85% of which were cases of thalassemia.[6,23,26] Iron overload is a major pathophysiological change in thalassemic patients and could be a factor that enhances host susceptibility to pythiosis, possibly by promoting infectivity of *P. insidiosum* or by compromising host immunity.[6,65–67] However, several pythiosis patients were reported with underlying paroxysmal nocturnal hemoglobinuria, which is associated with iron deficiency,[6] indicating that iron overload alone may not explain susceptibility to *Pythium* infection. Thalassemia and paroxysmal nocturnal hemoglobinuria share a common pathophysiological feature of chronic hemolysis. Identification of roles of iron and hemolysis as susceptibility factors for the host to pythiosis needs further investigation. In contrast to these forms of pythiosis, the majority of patients with ocular pythiosis (84% of cases) report no underlying condition, indicating that healthy individuals are susceptible to ocular infection.[6,29]

96.1.4 Diagnosis

Procedures are available to culture *P. insidiosum* from patient tissue using standard microbiological procedures and conventional media for the culture of fungi. While most clinical laboratories can attempt the culture of *P. insidiosum*, the method is time-consuming and laborious, often fails to isolate the pathogen, and requires expertise to identify *P. insidiosum* in successful cultures. As an alternative method, in-house immunological and molecular assays have been developed to facilitate diagnosis of pythiosis.[7,68–83] These alternative techniques are reliable and more convenient than the standard culture method. In addition, some of the serodiagnostic tests can be used to monitor anti-*P. insidiosum* antibody level to evaluate treatment response.[68,70–73,77] The following section provides a summary of the methods currently used for diagnosis of pythiosis.

96.1.4.1 Conventional Techniques

Conventional techniques for diagnosis of pythiosis include (i) isolation and identification of *P. insidiosum* from clinical specimens by standard culture techniques, (ii) detection of anti-*P. insidiosum* antibodies in patient serum samples, and (iii) direct detection of *P. insidiosum* in infected tissue.

96.1.4.1.1 Culture Identification

Culture identification is a simple microbiological method for the diagnosis of pythiosis by isolation of the causative agent, *P. insidiosum*, from a clinical specimen. *P. insidiosum* can be grown on various types of nutrient agar: Sabouraud dextrose agar, potato dextrose agar, corn meal agar, soil extract agar, Czapek-Dox agar, tissue culture medium, vegetable extract agar, peptone-yeast-glucose agar, 2% water agar, potato flakes agar, and blood agar.[20,84] *P. insidiosum* colonies on agar are flat with no aerial hyphae and white, yellow, or brown in color. De Cock et al. report that the optimal temperature for growth of the pathogen is between 34°C and 36°C, with minimum and maximum temperatures for growth of 10°C and 45°C.[1] Davis et al. report an optimal growth rate at 37°C.[85] Failure to isolate *P. insidiosum* from clinical specimens is associated with low temperature storage of clinical specimens prior to culture, as either refrigeration or freezing can kill the pathogen.[40,41] In our experience, attempts to isolate the pathogen from human specimens transported on ice were usually unsuccessful. Grooters et al. demonstrated that a higher success rate for isolation of *P. insidiosum* was achieved by culturing fresh specimens from affected horses directly on antibiotic-containing medium.[84] Therefore, specimens should be stored at room temperature during transportation to a clinical microbiology laboratory, and should be freshly cultured on antibiotic-containing media at room temperature or at 37°C. *P. insidiosum* grows relatively fast, as indicated by a growth rate of up to 12 mm/day, and macroscopic colony appearance in a day or 2.[20] To confirm the identity of the cultured organism as *P. insidiosum*, it is necessary to induce production of the characteristic zoospores. Chaiprasert et al. and Mendoza and Prendas describe methods for zoospore induction by culturing the pathogen on induction-medium agar containing sterile grass blades.[19,20]

96.1.4.1.2 Serodiagnostic Tests

Serodiagnostic techniques are convenient for diagnosis of pythiosis. The goal of these techniques is to detect anti-*P. insidiosum* antibodies as indirect evidence of *P. insidiosum* infection. Multiple immunological approaches have been employed for the development of diagnostic assays: immunodiffusion (ID),[68–70] enzyme-linked immunosorbent assay (ELISA),[71–73] Western blot,[74,75,78] immunochromatographic test (ICT),[76] and hemagglutination test (HA).[77] Antigen sources for development of these assays are prepared from crude extract of *P. insidiosum* hyphae (called soluble antigen from broken hyphae; SABH) or secretory antigens in culture medium (called culture filtrate antigen; CFA). These techniques vary in their sensitivity and specificity.

ID tests for the diagnosis and treatment monitoring of pythiosis in animals and humans were invented by Mendoza et al., Imwidthaya and Srimuang and Pracharktam et al.[68–70] The ID tests are agar-medium based and rely on the detection of precipitation lines generated by the specific reaction of *P. insidiosum* antigens (CFA) with anti-*P. insidiosum* antibodies (in patient serum). In the standard setup, each of the CFA and patient serum is added to 5 mm diameter wells that are 5 mm apart in agar medium (1% purified agar in Veronal buffer, pH 7.8; 1% phenolized agar; 2% water agar).[68,70,71] Diffusion of the CFA and serum occurs during overnight incubation in a moist chamber, and a precipitation line, observed by naked eye in the following days, indicates a positive result. The absence of the precipitation line is taken as a negative result. Although the test is highly specific, is easy to perform, can test serum from both humans and animals without any modification, and requires cheap and available reagents, the low sensitivity of detection leads to a high rate of false negative results and compromises the efficacy of this test.[71,76,77]

Increased sensitivity for detection of anti-*P. insidiosum* antibody is achieved by ELISA and Western blot techniques developed by several investigators, that is, Mendoza et al., Grooters et al., Krajaejun et al., Vanittanakom et al., and Supabandhu et al.,[71–73,78,79] using SABH or CFA as the antigen source for coating ELISA plates or blotting to a membrane. These techniques have very high sensitivity (up to 100%) and specificity (up to 100%) for diagnosis of pythiosis, and thus overcome the sensitivity problem of the ID test. However, these advantages of ELISA and Western blots assays are offset by their complexities. They are time-consuming and multistep procedures, use expensive reagents and equipment, and require trained personal to perform. These disadvantages limit the use of these assays in clinical diagnostic laboratories.

Recently, an ICT has been developed as a rapid and easy-to-use assay for diagnosis of human pythiosis.[76] In the ICT, CFA is spotted onto a nitrocellulose membrane strip as an antigen source for detecting anti-*P. insidiosum* immunoglobulin G (IgG) in serum samples. From the sample end of the ICT strip, human IgGs in the serum move upward by capillary action and form complexes with the rabbit anti-human IgG-colloidal gold conjugate antibody that is blotted close by. The complexes continue moving upward through the nitrocellulose membrane. If the immune complexes contain human anti-*P. insidiosum* IgG, they bind CFA and develop a purple signal at the detection end of the ICT strip. In contrast, if the immune complexes lack human anti-*P. insidiosum* IgG, they pass through the spotted CFA without developing a signal. Detection sensitivity and specificity of ICT are high. Its turnaround time is less than 30 min.

Another easy-to-use assay format based on HA has been recently developed for diagnosis of pythiosis.[77] Sensitivity and specificity of the HA test are as high as that of ELISA, Western blot, and ICT. CFA is used as an antigen source for coating of sheep red blood cells (SRC). SRCs serve as signal amplification for the specific antibody–antigen reaction. Anti-*P. insidiosum* IgG and IgM in a serum of a pythiosis patient bind CFA on the surface of SRC and form a HA (an aggregation of the red blood cells [RBCs]), which can be simply visualized by the naked eye. In contrast, if there is no anti-*P. insidiosum* antibodies in the serum, no HA is observed. Turnaround time for performing the HA test is ~1 h. An advantage over the other serodiagnostic tests is that HA test is a sensitive test that can be used for detection of

anti-*P. insidiosum* antibodies of both human and animal sera, because there is no need for changing the host-specific conjugate antibody.

In addition to their use in diagnosis, the above-mentioned serological tests can be applied for monitoring anti-*P. insidiosum* antibody levels over the course of treatment.[68,70–72,77] A decrease in the antibody level, in association with clinical improvement after surgical treatment, is an indicator for a good prognosis. A drawback of all serodiagnostic tests for pythiosis is that they are likely to fail to detect anti-*P. insidiosum* antibody in patients with ocular pythiosis. It is postulated that this results from the failure of the eye infection to induce an antibody response.[76,77] Therefore, serological tests in patients with an ocular infection should be used with caution because of the danger of a false negative result. On the other hand, if a serum from an ocular patient is positive for anti-*P. insidiosum* antibody, this may indicate the presence of an invasive ocular infection or multiple sites of *Pythium* infection.

As an alternative to antibody detection for the diagnosis of pythiosis, several investigators have developed immunochemical and immunofluorescent assays for direct detection of *P. insidiosum* in preserved infected tissue or in pure cultures.[2,8,82,86] Rabbit polyclonal antibodies raised against *P. insidiosum* crude extract (SABH or CFA) are used to stain paraffin-embedded tissue sections. In the assay developed by Mendoza et al., fluorescein isothiocyanate is conjugated to the rabbit anti-*P. insidiosum* antibody, which allows signal detection with a fluorescent microscope (direct assay),[2,86] while Brown et al. and Triscott et al. use a secondary bovine anti-rabbit IgG antibody, conjugated with peroxidase or streptavidin-biotin-peroxidase complex, to react with the primary anti-*P. insidiosum* antibody and in the presence of chromogenic peroxidase substrates to develop a colored precipitate visible under light microscopy (indirect assay). This histological assay has been used for years to confirm pythiosis in animals; however, its specificity has been brought into question by the appearance of cross reactivity (a false positive result) to other fungi, that is, *Conidiobolus* sp.[80]

96.1.4.2 Molecular Techniques

Molecular techniques are increasingly useful for the diagnosis of infectious diseases including mycoses because they are very specific and sensitive, and all required reagents and equipment are widely available at reasonable cost. For diagnosis of pythiosis, molecular techniques have been developed for the direct detection of *P. insidiosum* from pure culture or infected tissue. The molecular diagnostic assays are especially useful in a laboratory that lacks conventional microbiological and immunological methods or for suspected cases that tested negative for pythiosis by other means. The ribosomal RNA (rRNA) gene repeat (or rDNA region, containing 18S rRNA, ITS1, 5.8S rRNA, ITS2, 28S rRNA, and the intergenic spacer) is often used as a target for the development of molecular detection assays and it has several advantages: (i) it is a multicopy gene that provides abundant target sequences and therefore higher detection sensitivity; (ii) it has highly conserved areas (18S, 5.8S, and 28S rRNA portions), which allow the possibility of obtaining rDNA sequence in unidentified organisms by using primers designed from the rDNA sequence of closely related species; and lastly, (iii) it contains variable regions (ITS1, ITS2, and the intergenic spacer), which allow differentiation at the genus and species levels. Advances in molecular assays for pythiosis are summarized here.

96.1.4.2.1 Sequence Analysis

Badenoch et al. were the first group to report the use of DNA sequence analysis for identification and confirmation of *P. insidiosum* infection in a patient with pythiosis.[7] Their protocol used the fungal universal primers NS1 and NS2[87] (Figure 96.2, Table 96.1) to amplify 510 bp PCR product of the 18S rRNA portion from genomic DNA of the culture-isolated organism, which was initially suspected to be a zygomycete. Subsequent sequencing and BLAST searching analyses provided a clue that the causative organism was not a zygomycete, but rather an oomycete. Then, a 900 bp portion of the internal transcribed spacer (ITS), which includes ITS1, the 5.8S gene, and ITS2, was successfully amplified by using primers TW81 and AB28, which were previously used to amplify the ITS of the closely related genus *Phytophthora*[88] (Figure 96.2, Table 96.1). Sequence of the PCR product was obtained by using the *Phytophthora* primers S1, S2, S3c, S4, S5, and S6[88] (Table 96.1), and it was 99.0% identical to the ITS sequence of *P. insidiosum* (strain CBS777.84), indicating that the organism in question was *P. insidiosum*.

Using the same approach, Rivierre et al. identified the first case of pythiosis from Africa,[42] Vanittanakom et al. confirmed the identification of *P. insidiosum* isolated from human patients,[79] and Reis et al. diagnosed pythiosis in a horse.[43] Rivierre et al. amplified the 785 bp PCR product of

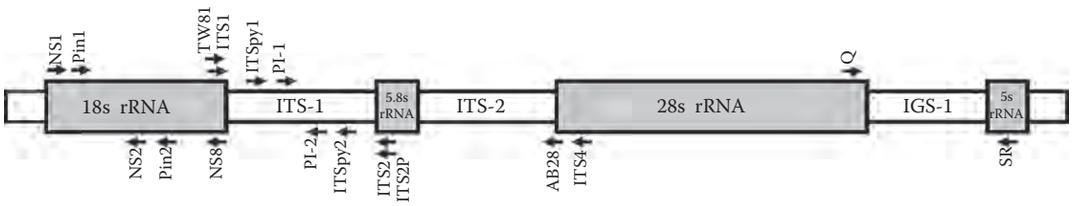

FIGURE 96.2 Map of the rDNA repeat of *P. insidiosum* showing primers (by names as indicated in the Table 96.1) and their estimated annealing sites. Arrows represent location and direction of each primer. Sizes of the rDNA repeat regions shown are not proportional to actual sizes. ITS, the internal transcribed spacer; IGS, the rDNA intergenic spacer.

TABLE 96.1
Primers for PCR Amplification of the *P. insidiosum* rDNA Repeat Region

Primer Name	Sequences	Tm (°C)	References
NS1	5′-GTAGTCATATGCTTGTCTC-3′	51.8	[7,87]
NS2	5′-GGCTGCTGGCACCAGACTTGC-3′	68.4	[7,87]
NS8	5′-TCCGCAGGTTCACCTACGGA-3′	64.8	[43,87]
ITS1	5′-TCCGTAGGTGAACCTGCGG-3′	63.5	[42,80,87]
ITS2	5′-GCTGCGTTCTTCATCGATGC-3′	61.4	[80,87]
ITS2P	5′-GCAGCGTTCTTCATCGATGT-3′	60.0	[81]
ITS4	5′-TCCTCCGCTTATTGATATGC-3′	56.2	[42,87]
Pin1	5′-TGGCTCTTCGAGTCGGGCAA-3′	65.1	[79]
Pin2	5′-GTCGGCATAGTTTATGGTTAAGA-3′	56.2	[79]
ITSpy1	5′-CTGCGGAAGGATCATTACC-3′	56.6	[79]
ITSpy2	5′-GTCCTCGGAGTATAGATCAG-3′	56.0	[79]
PI-1	5′-TTCGTCGAAGCGGACTGCT-3′	63.1	[80]
PI-2	5′-GCCGTACAACCCGAGAGTCATA-3′	63.4	[80]
Q	5′-ACGCCTCTAAGTCAGAATC-3′	55.1	[83]
SR	5′-GAAGCCCGGGTGCTGTCTAG-3′	64.3	[83]
TW81	5′-GCGGATCCGTTTCCGTAGGTGAACCTGC-3′	71.1	[7,88]
AB28	5′-GCGGATCCATATGCTTAAGTTCAGCGGGT-3′	68.9	[7,88]
S1	5′-CCGTAGGTGAACCTGCGGAGG-3′	66.4	[7,88]
S2	5′-GCACATCGATGAAGAACGCTG-3′	61.3	[7,88]
S3c	5′-GCGACTTCGGTTAGGACATT-3′	58.4	[7]
S4	5′-CCCGGAAGTGCAATATGCG-3′	60.5	[7,88]
S5	5′-GCCGAAGCCACCCATACCG-3′	62.5	[7,88]
S6	5′-GCTTAAGTTCAGCGGGTAATC-3′	56.8	[7,88]

the ITS portion from genomic DNA, extracted from infected tissue, using primers ITS1 and ITS4[87] (Figure 96.2, Table 96.1). Vanittanakom et al. amplified the 580 bp PCR product of the 18S rRNA portion from genomic DNA, extracted from pure culture, using primers Pin1 and Pin2[79] (Figure 96.2, Table 96.1). Reis et al. sequenced the 18S rRNA gene portion amplified by using the universal fungal primers NS1 and NS8[87] (Figure 96.2, Table 96.1). In each case, BLAST searching of the sequences of these PCR products successfully identified the organism as *P. insidiosum*.

AQ2

96.1.4.2.2 PCR Amplification

Use of PCR amplification, but without subsequent sequence analysis, is an alternative method for identification of *P. insidiosum* from tissue specimens and pure cultures (in the latter case, it is useful for bypassing the need for the difficult-to-achieve induction of zoospores to prove the identity of the isolated organism). A PCR assay for detection and identification of *P. insidiosum* was first described by Grooters and Gee.[80] They used the universal fungal primers, ITS1 and ITS2[87] (Figure 96.2, Table 96.1), for first-round amplification to determine the presence or absence of fungal DNA in the sample. The first-round amplification of *P. insidiosum* DNA produced a product of ~300 bp. The *P. insidiosum*-specific primers, PI-1 and PI-2[80] (Figure 96.2, Table 96.1), were used to generate a 105 bp product in the second-round nested-PCR amplification. The presence of this specific PCR product indicates that genomic DNA of the organism being tested contained *P. insidiosum* DNA. Grooters et al. showed that their nested-PCR assay is very sensitive and specific since the assay correctly detected all *P. insidiosum* isolates tested, and no nested-PCR products are observed for other *Pythium* species or for true fungi with similar morphology to *P. insidiosum*. Znajda et al. showed that, by substitution of primer ITS2 for primer ITS2P (Figure 96.2, Table 96.1), nested PCR can be used to detect *P. insidiosum* directly in frozen and ethanol-fixed infected tissue.[81] Thus, the nested PCR strategy is very useful in the detection of *P. insidiosum* as a means of diagnosing pythiosis, especially in cases in which the culture methods failed.

Vanittanakom et al. later developed a rapid single-round PCR amplification, which was used to identify *P. insidiosum* isolates in Thai patients with pythiosis.[79] Based on the ITS sequence of *P. insidiosum*, they designed two specific primers, ITSpy1 and ITSpy2 (Figure 96.2, Table 96.1), which were used to generate a single 233 bp PCR product in all four *P. insidiosum* isolates tested. Vanittanakom et al. went on to show that, when using this 233 bp ITSpy1–ITSp2 amplicon as a template, the *P. insidiosum*-specific primers for nested PCR (PI-1 and PI-2; Figure 96.2) of Grooters et al. failed to amplify *P. insidiosum*-specific fragment from one of the isolates. This result calls into question the universality of primers PI-1 and PI-2 for amplification of Thai isolates *P. insidiosum*. However, based on the limited number of

samples tested, there is no evidence indicating that the primers ITSpy1 and ITSpy2 of Vanittanakom et al. can amplify all *P. insidiosum* isolates from different geographic regions. Therefore, until there is an extensive evaluation of the specific primer sets of Grooters et al. and Vanittanakom et al., regarding detection sensitivity and specificity, tests for PCR amplification using either of them should be interpreted with caution because of the possibility of a false negative result.

Nested PCR has some advantages over a single-round PCR amplification in that it increases detection sensitivity by enriching the DNA target in the first round of amplification, and it increases detection specificity by restriction of annealing sequence targets of the nested primers to only the first-round PCR product, rather than to the whole genome (because of its complexity, the whole genome has the potential for more nonspecific annealing interactions and false positive PCR products). Although the two sets of amplification in nested PCR can make it a more sensitive procedure, the high sensitivity can be a disadvantage because of false positives resulting from cross contamination of *P. insidiosum* sample DNA in the steps of DNA extraction and amplification. Careful procedures should be performed for all PCR steps in order to prevent contamination. For example, use sterile containers and barrier tips for all steps, wear gloves, use a separate set of autopipettes, and use separate laboratory zones for DNA extraction, PCR amplification, and post-PCR gel electrophoresis. Inclusion of positive and negative control samples, along with the unknown sample, would help detect errors in the PCR procedure and false positive or negative results.

96.1.4.2.3 Species-Specific Probe

Schurko et al. have developed a dot-blot hybridization analysis of *P. insidiosum* genomic DNA, using a species-specific probe for the diagnosis of pythiosis.[83] The hybridization analysis employs a chemiluminescence detection system by which the signal from a positive hybridization is recorded on x-ray film. The rDNA intergenic spacer fragment (the sequence between 28S and 5S rRNA) of *P. insidiosum* is amplified from genomic DNA using primers Q and SR (Figure 96.2, Table 96.1) and a 530 bp species-specific probe is derived from it by HinfI enzyme digestion. The probe, which does not overlap any of the conserved rRNA regions and provides an intense hybridization signal, is used to hybridize denatured, extracted test-organism genomic DNA, which has been spotted (dot blotted) on a membrane. Schurko et al. demonstrated that their hybridization assay has high sensitivity, as it gives a positive signal for genomic DNAs of *P. insidiosum* isolates from all over the world, while high specificity is revealed by the lack of a hybridization signal for all genomic DNAs of other *Pythium* species or for true fungi. This method is clinically useful for identification of *P. insidiosum* DNA extracted from pure culture, and future applications of the method may make it possible to detect *P. insidiosum* DNA in infected tissue or patient blood. However, this technique is not routinely available for diagnosis of pythiosis because it requires a multistep procedure, expensive reagents, and a skilled personnel to perform the assay.

96.2 METHODS

This section describes, in the first part, several established sample preparation methods for extraction of genomic DNA of *P. insidiosum*. This is a very important step for the generation of a high-quality target DNA for the various downstream, conventional molecular diagnostic methods. In the second part, detection procedure guidelines are provided for using the extracted DNA in the identification and diagnosis of pythiosis, using standard molecular detection techniques.

96.2.1 Sample Preparation

P. insidiosum isolated from a clinical sample can be maintained on Sabouraud dextrose agar at 28°C–37°C with subculturing to a new agar plate every 2–4 weeks. Alternatively, the organism can be stored for months, as a few pieces of mycelial-attached agar, submerged in sterile water at room temperature. To prepare hyphae for DNA extraction, *P. insidiosum* from Sabouraud dextrose agar or sterile water storage is subcultured on Sabouraud dextrose agar for several days at 28°C–37°C to allow for active grow. Several small pieces of mycelial-attached agar pieces are excised from the edge of *Pythium* colony and transferred to 100 mL of Sabouraud dextrose broth for shaker culture (~150 rpm) for 5–10 days at 28°C–37°C. Hyphal growth is harvested by filtration onto sterile filter paper or cloth and washed once with 100 mL of phosphate-buffered saline (PBS). Hyphal material can be stored frozen at −80°C for future use. Several methods for DNA extraction of pure culture, frozen hyphae, and tissue are described in detail as follows.

96.2.1.1 Conventional Genomic DNA Extraction

The first method for genomic DNA extraction from pure culture and frozen hyphae of *P. insidiosum* is modified from the method used by Jackson et al.[89] and Pannanusorn et al.[90] To start the DNA extraction, fresh or frozen hyphal cells are placed in a precooled mortar in the presence of liquid nitrogen and are ground to a fine powder with a pestle. Then, the hyphal powder (200 mg) is placed in a 1.5 mL tube and 600 µL of lysis buffer (400 mM Tris–HCl [pH 8.0], 60 mM ethylenediaminetetraacetic acid [EDTA], 150 mM NaCl, 1% sodium dodecyl sulfate [SDS], and 40 mg/mL proteinase K) is added and mixed. The sample is incubated at 60°C for 60 min with occasional and gentle inversion. Then, 100 µL of 5 M sodium perchlorate is added and incubated at 60°C for 15 min. The sample is stored on ice, and DNA extraction is sequentially performed with 500 µL of ice-cold chloroform, with an equal volume of phenol-chloroform-isoamyl alcohol (volume ratio, 25:24:1) and finally with an equal volume of chloroform. Extracted genomic DNA is precipitated with 0.6 volume of isopropanol or 2 volumes of ice-cold absolute ethanol before washing two times with 500 µL of 70% ethanol, air-dried for 5–10 min, and resuspended in 100–200 µL of sterile water. Extracted DNA is stored at 4°C or −20°C.

An alternative method for extraction of genomic DNA from *P. insidiosum* is modified from the fungal DNA extraction method of Hogan and Klein.[91] First, extraction buffer (100mM Tris-HCl pH8.0, 100 mM EDTA, 250 mM NaCl, 100 µg/mL proteinase K, and 1% SDS [the latter two components should be added immediately before use]) is used to resuspend the hyphal powder in a 50 mL tube at the ratio of 5 mL of extraction buffer per 1 g hyphal power. The mixture is incubated at 55°C for 2 h before centrifugation at 10,000 × *g* at room temperature for 60 min to remove cell debris. An equal volume of phenol-chloroform-isoamyl alcohol (volume ratio, 25:24:1) is added to the supernatant, shaken hard to mix, allowed to sit for 5 min, and centrifuged at 2000 × *g* for 30 min to separate the upper aqueous and the lower organic phases. Two volumes of absolute ethanol or 0.6 volume of isopropanol are added to the aqueous phase and centrifuged at 2000 × *g* for 5 min to pellet the DNA. The pellet is washed with 70% ethanol and dissolved with 250–500 µL TE buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA pH 8.0) in a 1.5 mL tube. The sample is treated with RNaseA (50 µg/mL) at 37°C for 30 min, extracted with an equal volume of chloroform–isoamyl alcohol (volume ratio, 24:1), and centrifuged at 14,000 × *g* for 10 min. The resultant supernatant is precipitated with 0.1 volume of 3 M sodium acetate, pH 5.2, and with either 2 volumes of absolute ethanol or 1 volume of isopropanol and centrifuged at 14,000 × *g* for 10 min to pellet the DNA. The pellet is washed twice with 70% ethanol, air-dried for 10–15 min, and dissolved in 100–200 µL TE buffer. The DNA is allowed to sit overnight at 4°C before checking DNA recovery and integrity by absorbance at 260 and 280 nm and by 1% agarose gel electrophoresis.

96.2.1.2 Rapid Genomic DNA Extraction

The methods of Woods et al.[92] and Vanittanakom et al.[79] are modified for a rapid genomic DNA extraction from a pure culture of *P. insidiosum*. A few loops of *P. insidiosum* hyphae is spooled out from broth culture, suspended in a 0.5 mL volume of lysis buffer (250 mM Tris pH 8.0, 1.5% SDS), and boiled for 30 min in a water bath. The boiled-hyphal suspension is vortexed hard for 2 min, and then the DNA is extracted with an equal volume of phenol-chloroform-isoamyl alcohol (volume ratio, 25:24:1) and precipitated from the aqueous phase with 0.1 volume of 3 M sodium acetate (pH 5.2) and 2 volumes of absolute ethanol. The DNA pellet is air-dried, resuspended in 50–75 µL of sterile water, and the DNA concentration is estimated by electrophoresis of a 5 µL sample in 1% agarose gel or with a spectrophotometer (260/280 nm).

96.2.1.3 Genomic DNA Extraction from *P. insidiosum*-Infected Tissue

A method for genomic DNA extraction of *P. insidiosum* in frozen or ethanol-fixed tissues has been reported and successfully used by Znajda et al.[81] In brief, a piece of infected tissue (5 mm^3) is cut and placed in a 1.5 mL tube with 1000 µL digestion buffer (50 mM Tris–HCl [pH 7.2], 50 mM EDTA, 1% SDS, and 1% 2-mercaptoethanol) and 300 µg of proteinase K. The tissue is incubated at 65°C for 2 h before addition of another 300 µg of proteinase K. The tissue is vortexed, incubated at 65°C for 2–4 h, and centrifuged at 10,000 × *g* for 15 min at room temperature to collect the supernatant containing the genomic DNA. The supernatant is further extracted with an equal volume of chloroform-phenol-isoamyl alcohol (volume ratio, 25:24:1) and the sample is centrifuged as above. Genomic DNA in the aqueous phase is then precipitated by adding 1 volume of isopropanol in the presence of 0.3 M sodium acetate pH 5.2. The DNA pellet is washed twice with 70% ethanol, air-dried, resuspended in 100 µL sterile deionized water, and stored at –20°C for future use. DNA is diluted at 1:10 in sterile deionized water before using as a template in PCR.

96.2.2 Detection Procedures

96.2.2.1 Sequence Analysis for Species-Specific Identification

At least four regions of rDNA of *P. insidiosum* can be used for species-specific identification by sequence analysis. These four rDNA regions can be PCR amplified using genomic DNA as template together with the following primers: TW81 and AB28 (900 bp PCR product[7,88]), ITS1 and ITS4 (785 bp PCR product[42,87]), Pin1 and Pin2 (580 bp PCR product[79]), or NS1 and NS8[43,87] (Figure 96.2, Table 96.1).

Procedure

1. Prepare PCR mixture (20 µL) containing 10 ng genomic DNA, 0.1 mM deoxynucleoside triphosphates (dNTPs), 4.0 mM $MgCl_2$, 8 pmole of each primer, and 1 unit of Platinum *Taq* DNA polymerase (Invitrogen, United States) in 1× PCR buffer pH 8.4 (20 mM Tris-HCl, 50 mM KCl).
2. Perform the PCR amplification in a thermocycler using a cycling program consisting of a denaturation step at 95°C for 3 min; 30 cycles of 95°C for 1 min, 51°C (an annealing temperature for the primer ITS1 and ITS4) for 45 s, and 72°C for 1 min 45 s; and a final extension step at 72°C for 10 min. Assess the PCR product for amount and size by 1% agarose gel electrophoresis.
3. Perform direct sequencing reaction of the PCR product using the Big Dye Terminator Cycle Sequencing Kit version 3.1 (Applied Biosystems, United States). Prepare a sequencing mixture (10 µL) containing 4 µL sequencing reaction mix diluted 1:1 in sequencing buffer pH 9.0 (200 mM Tris, 5mM $MgCl_2$), 3.2 pmol primer and 200–500 ng template (PCR product from step 2). The reaction is performed in an ABI 2700 96 well plate thermal cycler with the following cycling temperatures: 10 s denaturation at 96°C, 5 s annealing at 50°C, and 4 min elongation at 60°C for a total of 25 cycles.
4. Precipitate the resulting product by adding 1 µL of 125 mM EDTA, 1 µL of 3 M NaOAc (pH 4.6), and 25 µL of absolute ethanol. Incubate the mixture for

15 min at room temperature before centrifugation at 2000 × *g* for 30 min at room temperature.
5. Wash the pellet with 70 µL 70% cold ethanol and centrifuge at 2000 × *g* for 15 min at room temperature. Add 12 µL HiDi formamide (Applied Biosystems) to each sample and sequence the product with the ABI 3100 Genetic Analyzer with 16 capillary tubes (Applied Biosystems, United States).
6. The sequence obtained is subjected to BLAST searching online, using the National Center for Biotechnology Information Nucleotide Database at the webpage, http://blast.ncbi.nlm.nih.gov/Blast.cgi. Definitive identification of *P. insidiosum* is based on 99%–100% sequence identity of the tested sequence to the previously described *P. insidiosum* 18S rRNA sequence.[7,42,43,79]

Note: Generally, the proper annealing temperature of other sets of primers can be estimated by a 5°C subtraction from the Tm of the lower of the two primers.

96.2.2.2 PCR Detection

The target for PCR amplification of *P. insidiosum* DNA has so far been limited to the region of rDNA repeat. The use of species-specific primers and the presence of a single PCR product of expected size in gel electrophoresis can be definitive for the identification of *P. insidiosum*, without the need for sequence analysis.

96.2.2.2.1 Single-Round PCR Protocol

The specific primer ITSpy1 and ITSpy2 (Figure 96.2, Table 96.1) are designed for a single PCR amplification of a specific product of 233 bp.[79]

Procedure

1. Prepare PCR mixture (50 µL) containing 10 ng genomic DNA template, 0.2 mM dNTPs, 10 pmol of each primer, 2.5 units of *Taq* DNA polymerase (Qiagen GmbH, Hilden, Germany), 20 mM Tris-HCl (pH 8.4), 50 mM KCl, and 1.5 mM $MgCl_2$.
2. Perform the PCR amplification in a thermocycler using a cycling program consisting of a denaturation step at 94°C for 3 min; 35 cycles of 94°C for 45 s, 60°C for 30 s, and 72°C for 30 s; and a final extension step at 72°C for 10 min.
3. Separate the PCR products on a 1% agarose gel and stain with ethidium bromide.

96.2.2.2.2 Nested PCR Protocol

The universal fungal primers ITS1 and ITS2 are used to enrich the DNA target in the first round of PCR amplification, which increases detection sensitivity. The nested primers PI-1 and PI-2 are designed to restrict annealing sequence area to only the first-round PCR product, which increases detection specificity.[80]

Procedure

1. For the first-round PCR amplification, prepare PCR mixture (50 µL) containing 10 ng genomic DNA template, 20 µM dNTPs, 20 pmol of each primer (ITS1 and ITS2), and 2.5 units of *Taq* DNA polymerase (Promega Corporation, Madison, WI), 10 mM Tris–HCl (pH 8.3), 50 mM KCl, and 1.5 mM $MgCl_2$.
2. Perform the first-round amplification in a thermocycler using a cycling program consisting of a denaturation step at 94°C for 3 min; 30 cycles of 94°C for 45 s, 64°C for 30 s, and 72°C for 30 s; and a final extension step at 72°C for 10 min.
3. For the second-round PCR amplification, prepare PCR mixture (50 µL) containing 5 µL of the first-round PCR product diluted at 1:50 in ddH_2O, 20 µM dNTPs, 20 pmol of each primer (PI-1 and PI-2), 2.5 units of *Taq* DNA polymerase (Promega Corporation, Madison, WI), 10 mM Tris–HCl (pH 8.3), 50 mM KCl, and 1.5 mM $MgCl_2$.
4. Perform the second-round amplification in a thermocycler using a cycling program consisting of a denaturation step at 94°C for 3 min; 15 cycles of 94°C for 30 s, 65°C for 30 s, and 72°C for 30 s; and a final extension step at 72°C for 10 min.
5. Separate the PCR products on a 1% agarose gel and stain with ethidium bromide.

96.3 CONCLUSIONS AND FUTURE PERSPECTIVES

Pythiosis can be a fatal infectious disease of humans and animals living in tropical and subtropical areas worldwide and is caused by a fungus-like organism, *P. insidiosum*. The disease has clinical features that mimic other infectious diseases caused by true fungi, such as aspergillosis and zygomycosis. The majority of health care professionals are not familiar with the disease, leading to underdiagnosis and misdiagnosis. The high rate of morbidity and mortality of pythiosis is exacerbated by delayed diagnosis and the lack of effective treatments. The ecological niche of *P. insidiosum* is aquatic or swampy areas, and direct contact of patients to its habitat is likely an initial step for acquiring the *Pythium* infection. Since natural habitats of *P. insidiosum* are widespread and the disease is devastating, increased attention and awareness of pythiosis should be promoted among health care providers.

Early diagnosis leads to a prompt management and a good prognosis of patients with pythiosis. Culture identification of *P. insidiosum* is difficult, laborious, and time-consuming, and it often fails to isolate the causative agent from clinical specimens. Serodiagnostic tests, that is, ID, ELISA, Western blot, ICT, and HA test are available, but use of these tests are limited because of the requirement of skilled personnel and specialized equipment and a technical limitation of detection sensitivity for some types of infection, that is,

early infection and ocular infection, which produce low or no antibody response. Direct detection of the pathogen in infected tissue by immunochemical assay should be a solution for the diagnostic problem relating to undetectable anti-*P. insidiosum* antibody. However, false positive reactivity of the immunochemical assay has been described in patients with *Conidiobolosis*, an infectious disease that clinicopathologically mimics pythiosis. Therefore, more still needs to be done, by way of developing new diagnostic techniques that have better sensitivity and specificity for pythiosis.

In recent years, molecular diagnostic assays have become useful techniques for facilitating diagnosis of many infectious diseases. Some molecular techniques, for example, sequence analysis, PCR amplification, and DNA hybridization using species-specific probe, have been developed for detection of *P. insidiosum* in pure culture and frozen ethanol-fixed infected tissues[7,42,43,79–81,83] and could solve diagnostic problems of conventional microbiological and immunological assays mentioned above. However, reliability of these molecular assays may be questioned because only a few *P. insidiosum*-infected and control samples were evaluated during testing for detection efficiency (i.e., sensitivity and specificity). The target gene for detecting *P. insidiosum* DNA has, so far, been limited to only rDNA sequence because genetic information of other target genes is lacking. The specific PCR primers, designed from the rDNA sequence, for detecting *P. insidiosum* isolated from one geographic area may not be able to detect all organisms isolated from other geographic areas.[34,35] Therefore, new assays must be developed in order to account for the apparent genetic diversity that is associated with geographic distribution and more completely cover all strains during diagnostic testing.

It is clear that molecular assays will be the most powerful techniques for diagnosis of pythiosis. Implementation of new molecular technologies, such as real-time PCR and loop-mediated isothermal amplification reaction,[93,94] for the development of better diagnostic tests for pythiosis has potential for rapid and highly reliable tests with a more user-friendly assay format. Two key aspects of establishing an efficient clinical assay, based on molecular techniques, are, first, identifying new gene targets with their corresponding sets of primers to create a new assay and, second, evaluating the new assay for a high level of specificity and sensitivity. It will be necessary to test a large number of pythiosis tissue samples harboring *P. insidiosum* strains from around the world and control samples representing a broad range of mycoses to ensure the reliability of the test. In addition to working with pure cultures and frozen ethanol-fixed infected tissues, diagnostic tests for pythiosis should also be established for paraffin-embedded tissues, as this would allow confirmation of earlier diagnoses and would also allow for retrospective studies of pythiosis using archival specimens.

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AUTHOR QUERIES

[AQ1] Please check if the edit to the sentence beginning "Released zoospores swim and adhere to …" is ok.
[AQ2] Author name "Reis et al." does not match with reference [87]. Please check.
[AQ3] Please check the book title in reference [87] for correctness.

TITLE: PCR Amplification of a Putative Exo-1,3-β Glucanase Gene for Identification of the Pathogenic Oomycete *Pythium insidiosum*

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RUNNING TITLE: Identification of *P. insidiosum* by PCR

KEYWORDS: *Pythium insidiosum*, Pythiosis, Oomycete, Diagnosis, PCR

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ABSTRACT

Background: *Pythium insidiosum* is the etiologic agent of pythiosis, a life-threatening infectious disease of humans and animals. Morbidity and mortality rates for pythiosis are high. Diagnosis of pythiosis is difficult and often delayed. The main treatment option for pythiosis is surgical removal of infected organ. Early diagnosis can lead to prompt treatment, and therefore, a better prognosis of patients with pythiosis. Diagnosis of pythiosis can be made by culture identification or serodiagnostic assays. Molecular diagnostic techniques are useful if microbiological and immunological assays are not available, or there is a suspected pythiosis case that tested negative by other methods. So far, PCR identification of *P. insidiosum* has been largely relied on amplification of the rDNA region.

Objective: Recently, we report a putative exo-1,3-β-glucanase gene (*PinsEXO1*), which encodes a specific immunogen of *P. insidiosum*. In the present study, we aims to design and evaluate diagnostic performance of *PinsEXO1*-specific primers, Dx3 and Dx4, for rapid single-round PCR identification of *P. insidiosum*, in comparison with the previously-reported rDNA-specific primers, ITSpy1 and ITSpy2.

Material and Methods: Genomic DNA (gDNA) from 35 *P. insidiosum* isolates and 48 control fungal organisms were prepared for diagnostic performance evaluation of the *PinsEXO1*- and rDNA-specific primers.

Results: When amplifying the control gDNA by using the Dx3/4 and ITSpy1/2 primer sets, no PCR product was observed, indicating that both primer sets had 100% detection specificity. When amplifying the *P. insidiosum* gDNA, the Dx3/4 primers provided an expected 550-bp amplicon for all 35 samples, while the ITSpy1/2 primers provided an expected 230-bp amplicon for only 32 samples. Thus, detection sensitivity of the Dx3/4 and ITSpy1/2 primer sets were 100% and 91%, respectively.

Conclusion: By using the Dx3/4 primers, *PinsEXO1* was an alternative, efficient, and novel PCR target for rapid single-round PCR identification of *P. insidiosum*.

INTRODUCTION

The aquatic, fungus-like, oomycete organism *Pythium insidiosum* is the etiologic agent of pythiosis, a life-threatening infectious disease of humans and animals living in tropical and subtropical areas of the world [1]. The natural habitat of *P. insidiosum* is swampy areas, such as, rice fields and ponds. *P. insidiosum* grows as a hyphae, and produces asexual zoospores which can disseminate to infect humans and animals [2]. Patients usually present with clinical features associated with either cutaneous infection (ulcerating skin lesions), vascular infection (gangrenous ulceration of extremities from arterial insufficiency), ocular infection (corneal ulcer), or infection of internal organs [1,3,4]. Morbidity and mortality rates for pythiosis are high. Diagnosis of pythiosis is difficult and often delayed. Conventional antifungal drugs are generally ineffective against *P. insidiosum*. The treatment of choice for pythiosis is extensive surgical removal of infected organ. Many patients died from an advanced infection.

Early diagnosis and prompt treatment can minimize the morbidity and mortality rates of pythiosis. Definitive diagnosis of pythiosis can be made by culture identification [5], immunohistological assays [6–8], and serodiagnostic tests [9–20]. Some limitations have been observed in these assays. For example, the culture identification technique is a time consuming, experience-required, and relatively-insensitive assay. Serodiagnostic tests usually fail to detect anti-*P. insidiosum* antibody in patients with ocular pythiosis. A tissue sample (i.e., paraffin-embedded tissue) was often the only available source of specimen for diagnosis of

pythiosis. In such case, diagnosis using an immunohistological assay is more suitable. However, the immunohistological assay requires a specific rabbit anti-*P. insidiosum* antibody, which is not routinely available. Moreover, cross reactivity with some pathogenic fungi (i.e., *Conidiobolus* and *Fusarium* species) compromises the detection specificity of the assay [7,21].

As an alternative assay, molecular diagnostic techniques, i.e., sequence homology analysis and PCR amplification, have been employed for detection of *P. insidiosum* from infected tissues or pure cultures [22]. The molecular target for identification of *P. insidiosum* has been largely relied on the rDNA region (also known as the ribosomal RNA (rRNA) gene repeat) [22]. The rDNA region presents in the genome of most eukaryotes, and comprises 18S rRNA, internal transcribed spacer 1 (ITS1), 5.8S rRNA, ITS2, 28S rRNA, and the intergenic spacer. The rDNA region is a popular diagnostic target because it is a multicopy gene and it contains variable regions (ITS1, ITS2, and the intergenic spacer) for differentiation at the species level [22].

The sequence homology analysis is a multi-step and time consuming procedure, comprising PCR amplification, DNA sequencing, and BLAST searching [18,23–26]. Some investigators preferably use rapid single-round [18] or nested [21,27,28] PCR for direct detection of *P. insidiosum*. Vanittanakom et al. [18] demonstrate that their ITSpy1 and ITSpy2 primers successfully amplified all four *P. insidiosum* Thai isolates tested, while the PI-1 and PI-2 primers, used by Znajda et al. [27], Grooters et al. [21], and Botton et al. [28], failed to amplify one Thai isolate. Thus, the ITSpy1/2 primers seem to be more efficient than the PI-1/2 primers for identification of the Thai isolates. However, an evaluation with extended number of both *P. insidiosum* isolates and fungal controls is needed to further determine diagnostic performance of the ITSpy1/2 primers.

Recently, we report a putative exo-1,3-β-glucanase gene (*PinsEXO1*), which encodes a specific immunoreactive protein of *P. insidiosum* [29]. The *PinsEXO1*-coding sequences of all 22 *P. insidiosum* isolates tested are conserved, suggesting that the gene can be a potential PCR target for *P. insidiosum* identification. In the present study, we aims to design and evaluate *PinsEXO1*-specific primers for rapid single-round PCR identification of *P. insidiosum*, in comparison with the rDNA-specific primer, ITSpy1 and ITSpy2.

MATERIALS AND METHODS

Microorganisms

Thirty-five clinical (n=31) and environmental (n=4) isolates of *P. insidiosum* were recruited for genomic DNA (gDNA) preparation. Identity of all *P. insidiosum* isolates was confirmed by culture identification and zoospore induction [5]. Forty-eight culture-proven isolates of various fungi (**Table 1**) from the Clinical Microbiology Laboratory, Department of Pathology, Faculty of Medicine, Ramathibodi Hospital, Mahidol University, were also collected for gDNA preparation (**Table 1**). All microorganisms were maintained on Sabouraud dextrose agar until use.

Genomic DNA Extraction

Extraction of *P. insidiosum* gDNA was performed using the modified methods of Aljanabi *et al.* [30]. Briefly, hyphal mat (~100 mg), harvested from 7-day culture in Sabouraud dextrose broth, was ruptured with glass beads (diameter, 710-1,180 μm; Sigma) in the presence of 400 μl of the salt homogenizing buffer (0.4 M NaCl, 10 mM Tris-HCl (pH8.0), 2 mM EDTA), using TissueLyzer MM301 (Qiagen, Germany). SDS (17 % (wt/vol); 45 μl) and proteinase K (20 mg/ml; 8 μl) were added to the cell lysate and incubated at 56 $^{\circ}$C for

overnight. NaCl (6 M; 0.3 ml) was then added. The mixture was briefly vortexed and centrifuged (10,000 x g for 30 min). Supernatant obtained was added to an equal volume of isopropanol, mixed, stored at -20 $^{\circ}$C for 1 hr, and centrifuged (10,000 x g for 20 min). The pellet was washed with 70% ethanol, air dried, and dissolved in 50-100 µl sterile water.

Extraction of gDNA of the fungal controls (**Table 1**) was performed using the modified method of Muller et al. [31] and Niu et al. [32]. Briefly, portions of hyphal colony (~50-100 mg) was harvested from active growing (5-7 days old) culture on Sabouraud dextrose agar, and transferred to a 1.7-ml tube. Six hundreds µl of the lysis buffer (100 mM Tris-HCl (pH 8.0), 50 mM EDTA, 500 mM NaCl, 0.2% 2-Mercaptoethanol (v/v), and 0.3% SDS) was added to each sample, vortexed vigorously with glass beads (diameter, 710-1,180 µm; Sigma), and incubated at 65 °C for 3 hr. The cell lysate was centrifuged at 10,000 x g for 10 min. DNA was extracted with an equal volume of phenol:chloroform (1:1). To precipitate DNA, 0.1 volume of 3M potassium acetate buffer (pH 5.2) and 2 volumes of absolute ethanol were added to the mixture, incubated at -20 °C for 30 min, and centrifuged at 10,000 x g for 10 min. The DNA pellet was then washed with 70% ethanol, dried, and resuspended in 40 µl of TE.

Concentration and purity of all DNA samples was estimated by measurement of optical density at 260 and 280 nm wavelengths using a NanoDrop® 2000 spectrophotometer (Thermo Scientific). All DNA samples were stored at -30 °C until use.

Polymerase Chain Reaction

The PCR amplifications were performed in a 25-µl mixture comprising 100 ng gDNA template, 10mM each primer, 1.5 mM $MgCl_2$, 0.2 mM dNTP mixture (Promega, USA), and 0.5 UI Taq-polymerase (Fermentas, USA) in 1X Taq-polymerase buffer. Amplification reactions were carried out in a Mastercycler-Pro thermal cycler (Eppendorf, USA) with the following setting: pre-denaturation at 94 °C for 3 min, 25 cycles of denaturation at 94 °C for 45 sec, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min, followed by the final extension at 72 °C for 7 min. Three pairs of primers were used to amplify all extracted gDNAs: (i) the fungal universal primer ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) [21]; (ii) the rDNA-specific primer ITSpy1 (5'-CTGCGGAAGGATCATTACC-3') and ITSpy2 (5'-GTCCTCGGAGTATAGATCAG-3') [18]; and (iii) the *PinsEXO1*-specific primer Dx3 (5'-GCGAGTTCTGGCTCGACTTTA-3') and Dx4 (5'-ACAAGCGCCAAAAAGTCCCA-3') designed by using the Primer-BLAST program [33], and the *PinsEXO1* accession number GU994093.1 [29]. A negative control (no template) was included in each round of PCR assays. The GeneRuler™ 100-bp plus DNA ladder (Fermentas, USA) was used as the molecular markers. Size and amount of each PCR product were analyzed by 1% agarose gel electrophoresis, and image captured by Molecular Imager® Gel Doc™ XR+ (Bio-Rad, USA).

DNA sequencing

The primer Dx3 and ITSpy1 were used to sequence *PinsEXO1*- and rDNA-derived amplicons, respectively. PCR products were purified using the NucleoSpinR Gel and PCR clean-up kit (NucleoSpin, USA). Direct sequencing was performed using the Big Dye Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems, USA). Automated sequencing was performed and analyzed using an ABI 3100 Genetic Analyzer and the Applied Biosystems Sequencing software (Applied Biosystems, USA). For sequence homology analysis,

each sequences obtained were blasted against the NCBI nucleotide database (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

RESULTS

The negative control (no template) and the ITS1/4 fungal universal primers were included for a quality check of the gDNA samples from all 83 organisms (**Table 1**). After PCR amplification with the ITS1/4, ITSpy1/2, and newly-designed Dx3/4 (sequences and annealing locations were shown in **Figure 1**) primers, the negative control did not provide any product by gel electrophoresis. The ITS1/4 primers successfully amplified rDNA from all gDNA samples tested (**Table 1**). When amplifying gDNA samples from the fungal controls (n=48; **Table 1**), the ITSpy1/2 primers (which target rDNA) and the Dx3/4 primers (which target *PinsEXO1*) provided no band. Thus, detection specificity of these primer sets were equally 100%.

When testing the gDNA samples, extracted from all *P. insidiosum* isolates, the ITSpy1/2 primers produced an intense band from 29 samples, a very faint band from 3 samples (the isolate P11, NAN06, and SIMI7695.48), and no band from 3 samples (the isolate P8, P13, and P21) (**Figure 2B**). The gDNA prepared from the *P. insidiosum* isolate P1, P22, and Pi-S provided a slightly smaller PCR product (~220 bp), compared to the calculated amplicon size (~230 bp) (**Figure 2B**). However, sequence homology analysis against the NCBI nucleotide database (BLASTN) showed that these amplicons matched the *P. insidiosum* rDNA. Overall, the ITSpy1/2 primers showed the detection sensitivity of 91.4%.

The Dx3/4 primers successfully amplified a single, relatively-intense, 550-bp PCR product from gDNA templates of all 35 *P. insidiosum* isolates tested (**Figure 2A**). Detection sensitivity of the Dx3/4 primers was therefore 100%. BLASTN search against the NCBI nucleotide database matched these amplicons to *PinsEXO1*. The lowest amount of gDNA template required for a successful PCR amplification (limit of detection) of *PinsEXO1*, using the Dx3/4 primers, was 1 ng.

DISCUSSION

Molecular diagnostic techniques for pythiosis are useful if microbiological and immunological assays are not available, or there is a suspected case that tested negative by other methods. So far, PCR identification of *P. insidiosum* has been largely relied on amplification of the rDNA region [22]. As an alternative assay, we evaluated diagnostic performance of the PCR primers, Dx3 and Dx4, which target *PinsEXO1* of *P. insidiosum* [29], in comparison with the previously-reported rDNA-specific primers, ITSpy1 and ITSpy2 [18]. The primer Dx3 and Dx4 were designed by using the well-established program called Primer-BLAST [33], which is publicly available at the NCBI website (http://www.ncbi.nlm.nih.gov/tools/primer-blast). Based on a given target DNA sequence (i.e., *PinsEXO1* gene), the Primer-BLAST program selects the most suitable primer set and uses the BLAST and global alignment algorithm to evaluate the selected primers for any cross annealing against all genes deposited in the NCBI nucleotide database. Thus, the resulting primers (Dx3 and Dx4) should be specific to the target sequence.

The PCR template was adequately present in all reactions, because the ITS1/4 universal primers can amplify an expected amplicon from all 83 gDNA samples included in this study. The no-template control (included in each round of PCR assays) was all read negative, indicating that there was no DNA contamination that could lead to a false positive result. To evaluate detection specificity, we collected 48 different culture-

proven fungal microorganisms (**Table 1**), as the controls in the PCR assay evaluation. We also included a number of *Aspergillus* spp. and *Zygomycetes*, because these fungi have microscopic features that could be confused with *P. insidiosum*. When the Dx3/4 and ITSpy1/2 primers were used to amplify gDNA prepared from the fungal controls (**Table 1**), no PCR product was observed by gel electrophoresis. This result indicated that there was no non-specific primer annealing to gDNA of the fungal controls, and thus, both primer sets equivalently had 100% detection specificity.

To evaluate detection sensitivity, gDNA prepared from all 35 culture-proven *P. insidiosum* isolates were PCR amplified by using the Dx3/4 and ITSpy1/2 primers. The ITSpy1/2 primers can amplify an expected amplicon from 32 out of 35 isolates tested, although three isolates (P11, NAN06, and SIMI7695.48) of which could be read negative due to a very faint PCR band (**Figure 2B**). In addition, gDNA of a few *P. insidiosum* isolates (P1, P22, and Pi-S) gave an unexpected smaller amplicon (~220 bp rather than ~230 bp), suggesting some variations in the rDNA region of *P. insidiosum* (**Figure 2B)**. Altogether, the detection sensitivity of the ITSpy1/2 primers was compromised, and thus, calculated to be 91%. On the other hand, based on the same set of gDNA samples, the Dx3/4 primers successfully amplified an expected intense PCR product (~550 bp) from all *P. insidiosum* gDNA samples tested (**Figure 2A)**. Therefore, the Dx3/4 primers had a better detection sensitivity (100%), when compared to that of the ITSpy1/2 primers (91%).

In conclusion, we demonstrated here that, when amplifying the gDNA templates of 35 *P. insidiosum* isolates and 48 fungal controls (**Table 1**), the ITSpy1/2 primers had excellent detection specificity (100%), but limited detection sensitivity (91%). By using the same set of templates for PCR amplification with the Dx3/4 primers, it was found that *PinsEXO1* was a more efficient PCR target (100% detection specificity and sensitivity) and it can be an alternative marker for molecular identification of *P. insidiosum*.

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TABLES AND FIGURES

TABLE 1: *Pythium insidiosum* isolates (n=35) and fungal controls (n=48) used for genomic DNA preparation in this study.

Microorganism	Number of isolates
Pythium insidiosum [a]	35
Cryptococcus neoformans	2
Penicillium marneffei	1
Candida species [b]	12
Aspergillus species [c]	3
Mucor species	3
Rhizopus species	2
Absidia species	1
Saksenaea species	1
Conidiobolus species	1
Basidiobolus species	1
Microsporum gypseum	1
Trichophyton species [d]	4
Trichosporon species [e]	2
Trichoderma species	1
Fusarium species	4
Curvularia species	1
Geotrichum species	1
Rhodotorula species	1
Torulopsis glabrata	2
Acremorium species	2
Scedeosporium apiospermum	1
Exophiala jeanselmei	1

Footnote:

[a] *P. insidiosum* isolated from patients with vascular pythiosis (n=14), patients with ocular pythiosis (n=10), patients with cutaneous pythiosis (n=3), patients with other forms of pythiosis (n=2); isolated from animals (n=2); and isolated from environment (n=4)

[b] *Candida* species: *C. albicans* (n=2), *C. tropicalis* (n=2), *C. parapsilosis* (n=2), *C. quilliermondii* (n=2), *C. rugosa* (n=1), *C. lusitaniae* (n=1), *C. laurenttii* (n=1), and *C. dublinensis* (n=1)

[c] *Aspergillus* species: *A. flavus* (n=1), *A. terrcus* (n=1), and *A. fumigatus* (n=1)

[d] *Trichophyton* species: *T. rubrum* (n=3), and *T. mentagrophytes* (n=1)

[e] *Trichosporon* species: *T. asahii* (n=1), and *T. mucoides* (n=1)

FIGURE 1: Sequences and annealing locations of the primer Dx3 (first gray box) and Dx4 (second gray box; reverse complement sequence) in reference to the putative exo-1,3-β-glucanase genes from *Pythium insidiosum* (Query sequence; Accession number, GU994093.1) and *Phytophthora infestans* (Subject sequence; Accession number, AF494014.1). The primer Dx3 and Dx4 perfectly anneal to the *P. insidiosum* glucanase gene, but failed to properly anneal to the *P. infestans* glucanase gene. Sequence alignment analysis of the glucanase genes from *P. insidiosum* and *P. infestans* (performing online at http://blast.ncbi.nlm.nih.gov/Blast.cgi) shows E-value of 4e-141, identities of 72%, and gap of 1%.

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Query  1     ACTACGGYAACCTGAACCAGAGTCTCACGGCGGTCGAGGAGATCGTGAAGCGGTACGCGA  60
Sbjct  1943  ACTACGCCAACCTGAACCACTCCATTGTGGCCGTGGAGGCTATTATCAACCGCTACAAGG  2002

Query  61    GCCACCCGGCGGTGCTCGGGCTGCAGCCCGTGAACGAGCCRTGGGAGCTGACRCCCATCA  120
Sbjct  2003  GCCACAAGGCTATCATGGGTCTCGAGCCGGTGAACGAGCCATGGGAACTCACTCCTATTA  2062

Query  121   AGGTGCTCAAGACGTACTACTGGAAGTCGTACAAGCGCGTGAAGGCGCTCGCGCCGCACT  180
Sbjct  2063  AGGTGCTGAAGCGGTACTACTGGAAGTCCTACAAACGTGTGAAGGTGTTGGCTCCCTCGT  2122

Query  181   GGAAGTTTGTGCTGCACGACTCGTTCCGCTTTGGCCGCGAGTTCTGGCTCGACTTTATGC  240
Sbjct  2123  GGAAATTCGTCATCCACGACTCGTTCCGCTTCGGTCTGCAGTTCTGGGCCAAGTTCCTCA  2182

Query  241   GCGGSTGCCCCGGACATTGCGATCGACACGCACATCTACCAGGCGTGGATGAACCCCGGCA  300
Sbjct  2183  AGGGCTGCCCCGATATCGCTCTGGACACGCACATCTACCAGGCCTGGAACCCTCCTGGAA  2242

Query  301   CGAAGGAGGACTTTTACTCGAACGCGTGCCAGCAAAAGTACACGATCACGGACATTGAGA  360
Sbjct  2243  CGGTTGCAGACTTCTTCTCCAACGCTTGTCAGCAGAAATATGTCATCAGTGACATGGAGA  2302

Query  361   ACGCCGTRATGCCCGTGATCGTCGGCGAGTGGTCGCTCGGCACCGACAACTGCGCGATGT  420
Sbjct  2303  ACGCCATGATGCCGGTGATTGTCGGCGAGTGGTCTATTGGCACGGACAACTGCGCCATGT  2362

Query  421   GGCTCAACGGCTTCAACGACAACCTCCCGGGCTTTCCCAAGGTCATCTGCCAGCTGCGCC  480
Sbjct  2363  GGCTCAACGGCTTCAACGACAACTTGCCTGGCTTCCCTAAGGTGCAGTGCCACATGATAG  2422

Query  481   ACTGCCCCGTCGAGAGCACGTACCTCGGCAAGGGGTTCCCGGGCACGCCGCTTGACACGA  540
Sbjct  2423  ACTGCCCGGTCAACAGTACGTACCTCGGCGACGGCTTCCCGGGCACTCCTTTGGACAAGA  2482

Query  541   CCAAGCCRATCCAGGGTCCCTACGGCACGGGCACGTCGGGTCCGAGCTTCGGYCTTTGCC  600
Sbjct  2483  CCAAGCCGATCCAGGGTCCATACGGCACTGGCATGTCTGGTCCGTCCTTCGGTAAGTGTC  2542

Query  601   CCGTGAACAGCAACCTCACGTTCGGCC-AGAAGACGCCCGAGGACGAGCTCAAGTTCATG  659
Sbjct  2543  CCGTTACCAGTCAGGACTCGTTCCACCAAGACGACG-ACGCGGCGTTGACCAAGTCGCTG  2601

Query  660   AAGAACCTCATGTCCAAGAAGCTCAA-CGCGTGGCTGCTCGGCCACGGCTTCTACTTCTG  718
Sbjct  2602  A-----CTC-TG---AAGAAGCTCAATGGCTTCGCTAAT-GGCCACGGCTGGTACTTCTG  2651

Query  719   GAACTTTAAGACGGAGCTCGACACGCGCTGGGACTTTTGGCGCTTGTGCGCGCCGGCGT  778
Sbjct  2652  GAACTTCAAGACGGAGTTTGCGACCAAGTGGAGCTTCCTGGACCTATGGCGTATGGGCGC  2711

Query  779   CATGCCCAAGAACATCTCGGACTATGACGACGCCGACGGCATCTTTGACGCGTGCGAGCG  838
Sbjct  2712  CTTCCCCAAGAACGTGTCGGACTACCACGAGAGCGATGGAGTGGAGAGAGCGTGTGTGAA  2771

Query  839   CGAGGACAAGGGCGACTTTGTGTGCCGCGCCAAGCGCGGCGTCAAGCCGTTTGAGCTCGA  898
Sbjct  2772  GGAAGACAGAGGCGAGTTCGTCTGCCGTGCCAAGCGTGGAGTCAAGGAGTTCGAGCTCAA  2831

Query  899   GAACGGACTCGCGTACGCGTGCAAC  923
Sbjct  2832  GAGCGGCCTCGCGTTCGCGTGCAAC  2856
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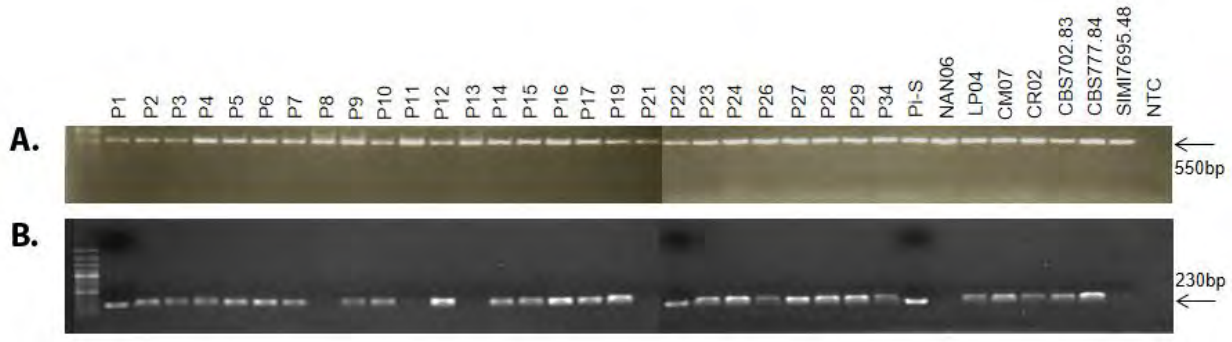
FIGURE 2: Gel electrophoresis showing the PCR products amplified from gDNA of the 35 *P. insidiosum* isolates by using: (**A**) the Dx3/4 primers, which target the putative exo-1,3-β-glucanase gene of *P. insidiosum* or *PinsEXO1* (Amplicon size, 550bp); and (**B**) the ITSpy1/2 primers, which target the rDNA region of *P. insidiosum* (Amplicon size, 230bp) (NTC, No-template control).



TITLE: Peptide ELISA for Antibody to *Pythium insidiosum* Based on Predicted Antigenic Determinants of Exo-1,3-β-Glucanase

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RUNNING TITLE: Peptide ELISA for Antibody to *P. insidiosum*

KEYWORDS: *Pythium insidiosum*; Pythiosis; Diagnosis; ELISA; Exo-1,3-β-Glucanase

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ABSTRACT

Human pythiosis is a life-threatening infectious disease caused by *Pythium insidiosum*. Morbidity and mortality levels are high. Main treatment option for pythiosis is surgical removal of infected organ. Early diagnosis leads to prompt treatment and better prognosis. Diagnosis of pythiosis relies on culture identification, serodiagnosis, and molecular-based assay. Serodiagnosis is more popular due to its efficient performance. However, preparation of a serdiagnostic test requires culture filtrate antigen (CFA), extracted from the live pathogen. Previously, we reported a specific 74-kDa immunoreactive protein of *P. insidiosum*, which is encoded by exo-1,3-β-glucanase gene (*PinsEXO1*). Because PinsEXO1 protein is recognized by sera from pythiosis patients, but not any control sera tested, this protein could be an alternative target used for detection of anti-*P. insidiosum* antibody. This study aims to: (i) identify, synthesize, and evaluate an antigenic determinant (epitope) of PinsEXO1 for serodiagnosis of pythiosis based on peptide ELISA, and (ii) compare diagnostic performance of the peptide ELISA and the established CFA-based ELISA. Two antigenic determinants of PinsEXO1 (Peptide-A and -B) were predicted by using the PREDITOP program. Sera from 34 pythiosis patients and 92 control subjects were included for assay evaluation. Peptide-A, Peptide-B, and CFA-based ELISAs provided equally high detection specificity (100%). While Peptide-B ELISA had limited detection sensitivity (91%) and accuracy (98%), both Peptide-A and CFA-based ELISAs showed equivalently high detection sensitivity (100%) and accuracy (100%). In conclusion, Peptide-A is a more efficient epitope than Peptide-B, and it can be an alternative antigen used for development of a serodiagnostic assay for pythiosis.

INTRODUCTION

Pythium insidiosum is the only aquatic, fungus-like, oomycetous microorganism, belonging to the Stramenopiles of the supergroup Chromalveolates, that causes a devastating infectious disease, called pythiosis, in both humans and animals, i.e., horses, dogs, and cattle (Mendoza *et al*, 1996; Kamoun 2003; Keeling *et al*, 2005). Morphologically, *P. insidiosum* looks like a filamentous fungus, but phylogenetically, it is far different from true fungi (Kwon-Chung 1994). *P. insidiosum* produces a special structure, called zoospore. The zoospore can swim to attach host surface, geminate into host tissue, and cause pathology (Mendoza *et al*, 1993).

The first report of human pythiosis was in 1985 (Krajaejun *et al*, 2006b). Afterward, more cases have been increasingly reported, mostly from Thailand (Krajaejun *et al*, 2006b). Four different clinical features of human pythiosis have been described: (i) cutaneous pythiosis affecting face or extremities as a granulomatous lesion and ulcer; (ii) vascular pythiosis affecting arteries resulting in blood vessel occlusion and gangrene; (iii) ocular pythiosis affecting eyes as corneal ulcer; and (iv) unusual form of pythiosis affecting other internal organs, such as, gastrointestinal tract and brain (Krajaejun *et al*, 2006b). Thalassemic hemoglobinopathy is predisposing factor (Sathapatayavongs *et al*, 1989; Krajaejun *et al*, 2006b). The morbidity and mortality levels of pythiosis are high (Krajaejun *et al*, 2006b). Delayed diagnosis and lack of effective treatment are healthcare concern. Conventional antifungal drugs are ineffective in fighting against the *P. insidiosum* infection. Treatment of choice is

extensive surgery of an infected organ, which leads to life-long handicaps. Many patients died from advanced and uncontrolled infection. Early diagnosis can lead to a prompt treatment and a better prognosis.

Definitive laboratory diagnosis of pythiosis can be made by culture identification (Mendoza and Prendas 1988; Chaiprasert *et al*, 1990), serodiagnosis (Krajaejun *et al*, 2002, 2009; Jindayok *et al*, 2009; Keeratijarut *et al*, 2009; Supabandhu *et al*, 2009; Chareonsirisuthigul *et al*, 2013), and molecular-based analysis (Badenoch *et al*, 2001; Grooters and Gee 2002; Vanittanakom *et al*, 2004; Botton *et al*, 2011). Among the established diagnostic methods, serodiagnosis is more popular due to its convenient procedure. Several serodiagnostic assays have been developed for diagnosis of pythiosis, including immunodiffusion (ID), enzyme-linked immunosorbent assay (ELISA), Western blot (WB), hemagglutination (HA), and immunochromatography (ICT) (Krajaejun *et al*, 2002, 2009; Jindayok *et al*, 2009; Supabandhu *et al*, 2009). Chareonsirisuthigul *et al* (2013) compare these assays and show that ICT and ELISA have highest diagnostic performance. HA and ID often provide false negative results. ICT is a rapid and easy-to-use assay. However, to develop ICT, and also WB, requires reagents and equipment not routinely available in clinical laboratories. Unlike other assays, ELISA results are unambiguously interpreted by an ELISA reader, which can prevent some interpretation error by laboratory personnel.

Preparation of the serodiagnostic assays requires crude protein mixture [such as, culture filtrate antigen (CFA), and soluble antigen from broken hyphae (SABH)] extracted directly from the live pathogen. Batch-to-batch variation, as well as, significant degradation of the crude protein extract notably lead to inconsistency of diagnostic performance of the assays. Alternative to the crude protein extract, synthetic peptides, containing a specific antigenic determinant (epitope), have been proven to be a useful for development of an immunoassay for detection of antibody against a variety of pathogens (Gómara and Haro 2007; Velumani *et al*, 2011). Additionally, peptides are not infectious materials, and can be commercially synthesized at high purity and unlimited amount.

Recently, we identified a specific 74-kDa immunoreactive protein in the crude extract of *P. insidiosum* by Western blot (Krajaejun *et al*, 2006a). By using proteomic and genetic approaches, we later found that this protein is encoded by a putative exo-1,3-β-glucanase gene (*PinsEXO1*) (Krajaejun *et al*, 2010). PinsEXO1 protein is recognized by all sera from patients with pythiosis, but not any control sera, tested (Krajaejun *et al*, 2006a, 2010). Thus, PinsEXO1 could be a novel antigenic target used for specific detection of anti-*P. insidiosum* antibody. In the current study, we aim to: (i) identify, synthesize, and evaluate an antigenic determinant of PinsEXO1 for serodiagnosis of pythiosis based on a peptide ELISA, and (ii) compare diagnostic performance of the peptide-based ELISA and the established CFA-based ELISA (Chareonsirisuthigul *et al*, 2013).

MATERIALS AND METHODS

Serum samples

For an evaluation of ELISA, a total of 34 pythiosis serum samples were collected from vascular (n=33) and cutaneous (n=1) pythiosis patients, diagnosed based on successful isolation of *P. insidiosum* from infected tissue (Chaiprasert *et al*, 1990), or detection of anti–*P. insidiosum* antibodies in sera (Pracharktam *et al*, 1991; Krajaejun *et al*, 2002, 2009; Jindayok *et al*, 2009). Control samples (n=92) comprised: (i) serum samples from

healthy blood donors who came to the Blood Bank Division, Ramathibodi Hospital (n=56); (ii) serum samples from thalassemic patients without pythiosis (n=6); (iii) serum samples from patients with positive antinuclear antibody (n=9) and rheumatoid factor (n=2); and (iv) serum samples from patients positive for other infectious diseases (zygomycosis, 2; aspergillosis, 1; candidiasis, 1; histoplasmosis, 1; cryptococcosis, 1; syphilis, 2; anti-human immunodeficiency virus antibody, 2; anti-hepatitis B virus, 6; and anti-hepatitis C virus, 3). Positive and negative control serum samples were also included in the evaluation. All serum samples were kept at -20°C until used.

Preparation of culture filtrate antigen

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

Epitope prediction and biotin-labelled peptides

A partial sequence of the putative exo-1,3-β-glucanase encoding gene (*PinsEXO1*) of *P. insidiosum* (accession number: GU994093.1) (Krajaejun *et al*, 2010) was BLAST searched against a local transcriptome database of this pathogen (unpublished data). A resulting longer gene sequence was subject to protein translation using the ESTscan program (Iseli *et al*, 1999), and then, epitope prediction using the PREDITOP program (the program is based on turn predictions, and the peptides chosen are invariably very hydrophilic) (Pellequer and Westhof 1993). Peptides with predicted antigenic determinant were synthesized (>95% purity) and biotin-labelled by PEPNOME limited (China).

CFA-based ELISA

CFA-based ELISA was performed, using the method of Chareonsirisuthigul *et al* (2013), with some modifications. Briefly, a 96-well polystyrene plate (Costar, USA) was coated with 100 μl of CFA (5 μg/ml) in 0.1 M carbonate buffer (pH 9.6) and incubated at 4°C overnight, and then washed four times with phosphate buffer solution (pH 7.4) containing 0.5% Tween20 (PBS-T). The CFA-coated plate was blocked with 100 μl of casein buffer [1% casein in phosphate buffer solution (pH 9.6)] at 37°C for 1 hr, and washed four times with PBS-T. A test serum sample diluted 1:800 in the casein buffer was added to each well (100 μl/well), incubated at 37°C for 2 hr, and washed four times with PBS-T. The positive and negative control sera were tested in parallel. Each serum sample was tested in duplicate. Horseradish peroxidase-conjugated goat anti-human immunoglobulin G (Jackson immuno research, USA) diluted 1:100,000 in the casein buffer was added to each well (100 μl/well), incubated at 37°C for 1 hr, and washed four times with PBS-T. Freshly-prepared chromogen [20 μl of 0.6% TMB

and 1 ml of 0.009% hydrogen peroxide in acetate buffer solution (25 µmol/ml)] was added to each well (100 µl/well) and incubated at room temperature for ~5 min. The enzymatic reaction was stopped with 100 µl of 0.3 N sulfuric acid.

Peptide-based ELISA

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

Measurement of ELISA signal and statistical analysis

Optical density (OD) of each sample was measured using an Infinite 200 Pro ELISA reader (Tecan, Austria) at the wavelength 595-nm. Mean OD value of each sample was corrected for the OD of the buffer control (the casein buffer). The OD value of each serum sample was divided by the OD value of the same negative control serum, to obtain an ELISA value (EV). Sensitivity [which is “True positive (TP) / (False negative (FN) + TP) x 100”], specificity [which is “True negative (TN) / (False positive (FP) + TN) x 100”], accuracy [which is “(TP + TN) / (TP + TN + FP + FN) x 100], mean value of EV, and standard deviation (SD) were calculated using the Microsoft EXCEL2013 program.

RESULTS

Antigenic determinant prediction of *P. insidiosum* glucanase

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

Diagnostic performance of CFA- and peptide-based ELISAs

To ensure accurate result interpretation, all ELISA analyses included the same positive and negative control sera. Based on all pythiosis sera tested (n=34), mean EVs of the CFA-based, Peptide-A, and Peptide–B

ELISA were 145.9 (range, 16.1-236.9; SD, 57.6), 7.7 (range, 2.8–11.7; SD, 2.5), and 2.3 (range, 0.7–5.4; SD, 1.0), respectively. Based on all control sera tested [including serum samples from healthy blood donors (n=56), from thalassemic patients without pythiosis (n=6), from patients with autoimmune diseases (n=11), and from patients with other infectious diseases (n=19)], mean EVs of the CFA, Peptide-A and Peptide–B ELISA were 2.2 (range, 0.0–10.8; SD, 2.1), 0.9 (range, 0.2–2.3; SD, 0.6), and 0.4 (range, 0.1–1.2; SD, 0.3), respectively. Five cutoff points were calculated based on summation of the mean EV of control sera and 2, 3, 4, 5 and 6 SDs, respectively. Each cutoff point provided different detection sensitivity and specificity (**Table 1)**. An optimal cutoff EV was selected based on the highest accuracy obtained (**Table 1**). The summation of mean and 5 SDs (EV = 12.7) was the optimal cutoff value for CFA-based ELISA (assay accuracy = 100%). The summation of mean and 3 SDs was selected as an optimal cutoff value for both Peptide-A (EV cutoff = 2.7; assay accuracy = 100%) and Peptide-B (EV cutoff = 1.3; assay accuracy = 98%) ELISAs. Based on the optimized cutoff values, detection specificity of CFA, Peptide-A, and Peptide-B ELISAs were equally high (100%). While the CFA and Peptide-A ELISAs provided 100% detection sensitivity, the Peptide-B ELISA provided lower detection sensitivity (91%).

DISCUSSIONS

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

Because a peptide is a short stretch of amino acids, it could be poorly coated onto an ELISA plate, or it could adhere to plastic surface in a way that masks its antigenic epitope. This could lead to limited interaction of the peptide and its corresponding antibody. To solve this obstacle, each of Peptide-A and Peptide-B was conjugated to biotin. The biotin is efficiently and specifically captured by avidin, a molecule pre-linked on an ELISA plate. This strategy could enhance an efficiency of ELISA plate coating, and therefore, promote peptide-antibody interaction. Peptide-A and Peptide-B ELISAs were evaluated against a total of 34 pythiosis and 92 control sera. Both assays can discriminate EVs of the pythiosis sera from EVs of the control sera. However, overall diagnostic performance of Peptide-A ELISA (100% sensitivity, specificity, and accuracy) was better than that of Peptide-B ELISA (91% sensitivity, 100% specificity, and 98% accuracy). Although the epitope prediction program (i.e., PREDITOP) could increase chance to successfully obtain a peptide candidate with an antigenic

epitope, it cannot guarantee that all predicted epitopes (i.e., Peptide-A and Peptide-B) are efficient for antibody production or binding. Thus, extensive evaluation of more than one peptide is crucial and necessary. As seen here, Peptide-A is a more efficient epitope than Peptide-B. We have collected the pythiosis sera as many as possible for the assay evaluation. However, because the disease is relatively rare, the number of sera from pythiosis patients was limited (n=34). Further evaluation using more pythiosis sera will be useful to more accurately address the detection sensitivity of the assays.

The CFA-based ELISA has been recently developed and becoming a reference assay for serodiagnosis of pythiosis (Chareonsirisuthigul *et al*, 2013). Diagnostic performances of the Peptide-A ELISA and the CFA-based ELISA were compared. It was notable that the mean EVs of the pythiosis sera determined by the CFA-based ELISA (EV = 145.9) was 19-fold higher than that by the Peptide-A ELISA (EV = 7.7). However, there was slightly different (2.4 folds) for the mean EVs of the control sera determined by both assays. This may be due to the presence of hundreds of different antigenic epitopes in the CFA protein mixture, compared to only one epitope in the Peptide-A. Nevertheless, both assays provided equivalently-high diagnostic performance (100% sensitivity, specificity, and accuracy; **Table 1**).

In conclusion, we report here the use of synthetic PinsEXO1 peptides for serodiagnosis of human pythiosis. The detection specificity of CFA-based, Peptide-A, and Peptide-B ELISAs were equally high (100%). While Peptide-B ELISA had limited detection sensitivity (91%) and accuracy (98%), both Peptide-A and CFA-based ELISAs showed equivalently high detection sensitivity (100%) and accuracy (100%). Thus, the synthetic Peptide-A can be used as an alternative and efficient antigen for development of a serodiagnostic test for pythiosis. Synthetic peptide could address the concern on the batch-to-batch variation, as well as, significant degradation of the crude protein extract. Since peptides are non-infectious and commercially-available, they can be safely handled and prepared for use in a routine diagnostic laboratory.

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TABLES AND FIGURES

Table 1: Diagnostic performance of CFA- and peptide-base ELISA for detection of anti-*P. insidiosum* antibody in serum samples from 34 proven cases of human pythiosis, and 92 controls (CFA, culture filtrate antigen; ELISA, enzyme-linked immunosorbent assay; EV, ELISA value; SD, standard deviation).

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

Footnote:

[a] Mean ELISA value (EV) and standard deviation (SD) of the control sera (n=92)

[b] Sensitivity = true positive/(false negative+true positive) x 100

[c] Specificity = true negative/(false positive+true negative) x 100

[d] Accuracy (ability of an assay to provide true positive and true negative results) = (true positive+true negative)/(true positive+true negative+false positive+false negative) x 100

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

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

TITLE: Efficiency Comparison of Three Methods for Extracting Genomic DNA of the Pathogenic Oomycete *Pythium insidiosum*

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RUNNING TITLE: DNA Extraction of *P. insidiosum*

KEYWORDS: DNA extraction; Genomic DNA; Oomycete; Pythiosis; *Pythium insidiosum*

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ABSTRACT

Background: The fungus-like organism *Pythium insidiosum* is the causative agent of the life-threatening, tropical infectious disease, pythiosis, which has high rates of morbidity and mortality. A lack of reliable diagnostic tools and effective treatments for pythiosis presents a major challenge to healthcare professionals. Unfortunately, surgical removal of infected organs remains the default treatment for pythiosis. *P. insidiosum* is an understudied organism. In-depth study of the pathogen at the molecular level could lead to a better means of infection control. High quality genomic DNA (gDNA) is needed for molecular biology-based research and application development, such as: PCR-assisted diagnosis, population studies, phylogenetic analysis, and molecular genetics assays.

Objective: To evaluate quality and quantity of the *P. insidiosum* gDNA extracted by three separate protocols intended for fungal gDNA preparation.

Material and Method: Seven *P. insidiosum* isolates were subject to gDNA extraction by using conventional-extraction, rapid-extraction, and salt-extraction protocols.

Results: The conventional protocol afforded the best gDNA in terms of quality and quantity, and could be scaled up. The rapid-extraction protocol had a short turnaround time, but quality and quantity of the gDNA obtained were limited. The salt-extraction protocol was simple, rapid, and efficient, making it appealing for high throughput preparation of small-scale gDNA samples. In contrast to the other two methods, the salt-extraction protocol does not require the use of hazardous and expensive materials such as phenol, chloroform, or liquid nitrogen.

Conclusion: gDNA is required for many molecular studies of *P. insidiosum*. Determination of which gDNA extraction protocol is more suitable for a given experiment depends on the quality and quantity desired, the availability of materials and equipment, and allowable turnaround time.

INTRODUCTION

Pythium insidiosum is an aquatic, fungus-like, oomycete microorganism that causes a life-threatening infectious disease, called pythiosis, in humans and animals living in tropical and subtropical areas of the world (1). *P. insidiosum* inhabits swampy areas where it colonizes water plants (2). The organism generates a specialized structure, called a zoospore (3). When a zoospore contacts a human or an animal, it can invade host tissue and initiate an infection characterized by high rates of morbidity and mortality (4). Diagnosis of pythiosis is difficult and conventional antifungal drugs are ineffective. Extensive surgical removal of infected tissues (typically eyes or legs) is the primary treatment option for pythiosis. The dearth of reliable diagnostic tools and effective treatments remains a severe healthcare problem for pythiosis.

P. insidiosum is relatively understudied. Information on biology, evolution, and pathogenesis of *P. insidiosum* is lacking. A more in depth study of *P. insidiosum* at the molecular level could lead to a better understanding of the disease and thus better infection control and management. High quality genetic material (DNA and RNA) is required for a wide variety of molecular-based experiments and applications, such as: genome sequencing, molecular genetics study, PCR-assisted diagnosis, population genetic study, and phylogenetic analysis. Genomic DNA (gDNA) of *P. insidiosum* may be prepared by several methods (5–7). For example, Vanittanakom *et al.* have developed a rapid gDNA extraction protocol (rapid-extraction) for molecular identification of *P. insidiosum* (7). Chaiprasert *et al.* (6) and Pannanusorn *et al.*(5) have modified the conventional gDNA extraction protocol (conventional-extraction) of Jackson *et al.* (8) for molecular typing of some *P. insidiosum* strains. Information on quality and quantity of the gDNA extracted by these methods has not been published.

Aljanabi *et al.* report the universal and rapid salt-extraction protocol for isolation of gDNA (9). Their method provides high quality gDNA, suitable for PCR-based experiments. Unlike the other methods, the gDNA extraction protocol of Aljanabi *et al.* does not require phenol, chloroform and liquid nitrogen, which are expensive and environmentally hazardous reagents. In the present study, we aim at modifying the protocol of Aljanabi *et al.* (9) for extraction of *P. insidiosum* gDNA. We also aim at comparing the quality of gDNA extracted by the modified protocol of Aljanabi *et al.* (salt-extraction) with that of the rapid-extraction (7) and the conventional-extraction (5,6) protocols.

MATERIALS AND METHODS

Microorganisms and culture condition

Seven *P. insidiosum* isolates from patients with pythiosis (n=4), animals (n=2) and environment (n=1) (Isolate T1-7; **Table 1**) were recruited in this study. All isolates were confirmed by culture identification with zoospore induction (3,10). The microorganisms were sub-cultured on Sabouraud dextrose agar once a month. For preparation of large-scale gDNA, 10 small plugs (0.5 x 0.5 cm) of 7-day old mycelium-attached agar were transferred to 100 ml of Sabouraud dextrose broth, and incubated with shaking at 37°C for 10 days. For preparation of small-scale gDNA, *P. insidiosum* hyphae were cultivated in a Petri dish with 10 ml of Sabouraud dextrose broth and incubated at room temperature for 10 days. All mycelial mats were harvested, washed with distilled water, filtrated through filter paper (Whatman No.1), and stored at -30°C until use.

DNA extraction

The harvested mycelial mats of *P. insidiosum* were subjected to gDNA extraction by three different protocols: conventional-extraction, rapid-extraction, and salt-extraction. The conventional-extraction protocol was performed using the modified method of Jackson *et al.* (5,6,8). Briefly, 200-1,000 mg of mycelia were ground to fine powder in the presence of liquid nitrogen. The powder was transferred to a 50-ml conical tube. Twenty ml of extraction buffer [100 mM Tris-HCl (pH 8.0), 100 mM EDTA, 250 mM NaCl, 40 µg/ml proteinase K and 1% SDS] per 1 g of the mycelial power was added to the tube. The mixture was incubated at 55°C overnight. DNA was extracted with phenol:chloroform:isoamyl alcohol (25:24:1), followed by chloroform:isoamyl alcohol (96:4). An equal volume of ice-cold isopropanol was used to precipitate DNA. The DNA pellet was washed with 70% ethanol, air dried, dissolved in 500 µl TE buffer, and treated with RNase for 1 hr (final concentration, 50 µg/ml). The DNA sample was further extracted with chloroform:isoamyl alcohol (96:4), and precipitated with 0.1 volume of 3M sodium acetate and one volume of ice-cold isopropanol. The pellet was washed twice with 70% ethanol, air dried, and dissolved in 50-100 µl TE buffer.

The rapid-extraction protocol was performed according to the method of Vanittanakom (7). Briefly, 0.5 ml of lysis buffer [1.5%SDS and 0.25 M Tris (pH 8.0)] was added to 50-100 mg of harvested mycelia, boiled for 30 min, and vortexed for 2 min. DNA was then isolated with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) and precipitated with absolute ethanol. A DNA pellet was air dried and dissolved in 50 µl TE buffer.

The salt-extraction protocol was performed using the modified methods of Aljanabi *et al.* (9). Mycelial mats (50-100 mg) were transferred to a sterile microtube containing glass beads (diameter, 710-1,180 µm; Sigma) and 400 µl of the salt homogenizing buffer [0.4 M NaCl, 10 mM Tris-HCl (pH8.0), 2 mM EDTA] and homogenized using TissueLyzer MM301 (Qiagen, Germany) with the following setting: 2 min at 30 Hz. Forty-five µl of 17 % SDS and 8 µl of 20 mg/ml proteinase K were added to the cell lysate, gently mixed, and incubated at 56 $^{\circ}$C for 2 hr (or overnight). Next, 0.3 ml of 6 M NaCl was added to the sample. The mixture was vortexed for 30 sec, and centrifuged (10,000 x g) for 30 min. Supernatant was collected, mixed with an equal volume of isopropanol, and kept at -20 $^{\circ}$C for 1 hr. The sample was centrifuged (10,000 x g) at 4 $^{\circ}$C for 20 min. The pellet was washed with 70% ethanol, air dried, and dissolved in 100 µl sterile water.

Concentration and purity of all DNA samples was estimated by measurement of optical density at 260 nm and 280 nm wavelengths using a NanoDrop® 2000 spectrophotometer (Thermo Scientific) and by fluorescence-based measurement using a Qubit® 2.0 fluorometer (Invitrogen). DNA integrity was evaluated by 1% agarose gel electrophoresis. All DNA samples were stored at -30 °C until use.

Polymerase chain reaction

PCR amplification of several *P. insidiosum* genes was performed. The target genes included ribosomal DNA gene (*rDNA*; accession number, AY486144.1), a putative exo-1,3-beta glucanase-encoding gene (*EXO1*; accession number, GU994093.1) (11), and a putative RXLR effector-encoding gene (*UN04715*; submitted to the DNA Data Bank of Japan under accession number FX531705) (**Table 1**). Two sets of primers were used to amplify 233-bp (Primers: ITSpy1 and ITSpy2) and 931-bp (Primers: ITS1 and ITS4) amplicons from *rDNA* (**Table 1**). The primers Dx3 and Dx4 were used to amplify a 550-bp amplicon from *EXO1* (**Table 1**). The primers RXLR1

and RXLR2 were designed to amplify *UN04715* with a projected amplicon size of 1,575 bp (**Table 1**). PCR amplification was carried out in a reaction volume of 50 µl, containing 5 pmol each of forward and reverse primers, 1.25 U of *Taq* polymerase (Fermentas), 0.2 mM deoxynucleotide triphosphate mixture, 10 mM Tris-HCl (pH 8.8) with 50 mM KCl, 1-2 mM $MgCl_2$, and 100-200 ng of DNA template (**Table 1**). A hot start PCR protocol was performed with an initial denaturation at 95 °C for 3-6 min, followed by 25-30 cycles of denaturation (95 °C) for 30-45 sec, annealing (50-60 °C) for 30 sec, and extension (72 °C) for 30-120 sec (**Table 1**). The amplification reaction was finished with a final extension (72°C) for 10 minutes. Amplicons were analyzed by 1% agarose gel electrophoresis, in which 5-10 µl of each PCR product was loaded in loading buffer. Gels were stained with ethidium bromide and visualized by Gel Doc XR+ (Bio-Rad, USA).

RESULTS

Quality and quantity of extracted gDNA

Based on fluorescence measurements, the conventional-extraction protocol provided the highest DNA concentration (mean, 323 ng/µl; range, 121-540), followed by the salt-extraction (mean, 114 ng/µl; range, 39-199) and the rapid-extraction (mean, 46 ng/µl; range, 27-79) protocols (**Table 2**). However, the salt-extraction protocol provided the highest total yield (DNA obtained / mass of mycelium) (mean, 145 ng/mg; range, 47-260), followed by conventional-extraction (mean, 100 ng/mg; range, 28-183) and rapid-extraction (mean, 65 ng/mg; range, 39-136) (**Table 2**). A260/A280 ratios of the DNA extracted by the conventional-extraction protocol (~1.8–1.9) were lower than the ratios of the other two protocols (~2.0–2.1) (**Table 2**). DNA integrity was evaluated by agarose gel electrophoresis. High molecular weight DNA (size > 23kb) was observed in all samples prepared by the conventional- and salt-extraction protocols (**Figure 1**). In contrast, this high molecular weight DNA was absent in all samples prepared by the rapid-extraction protocol (**Figure 1**). Degraded DNA (characterized as a smear of low molecular weight material) was minimal in gDNA extracted by the conventional-extraction protocol, noticeable in gDNA extracted by the salt-extraction protocol, and prominent in gDNA extracted by the rapid-extraction protocol.

PCR amplification of target genes

When using the gDNA templates prepared by the conventional-extraction protocol, all primer sets (ITSpy1/2, ITS1/4, Dx3/4, and RXLR1/2; **Table 1)** successfully amplified target genes: *UN04715* (**Figure 2A**), *rDNA* (**Figure 2B and D**), and *EXO1* (**Figure 2C**). When testing gDNA templates prepared by the salt-extraction protocol, all primer sets produced relatively intense bands except for the primers RXLR1/2, which provided faint bands for the T3 and T6 templates (**Figure 2A**). Most of the gDNA templates prepared by the rapid-extraction protocol produced faint bands using primers ITSpy1/2 (**Figure 2D**). The Dx3/4 primers amplified the expected amplicon from five out of seven templates tested (**Figure 2C**); the ITS1/4 primers successfully amplified a product from only the T1 and T2 templates (**Figure 2B**); and the RXLR1/2 primers failed to produce an amplicon from all templates tested (**Figure 2A**).

DISCUSSION

In the present study, conventional-extraction, salt-extraction, and rapid-extraction protocols were used to extract the gDNA of *P. insidiosum*. These protocols were characterized with regard to efficiency and suitability of the gDNA produced for downstream applications. The high A260/A280 ratio (~1.8-2.1) of the gDNA extracted by all three methods indicates minimal protein contamination (Glasel 1995) (**Table 2**). DNA concentrations reported by UV absorbance measurement (NanoDrop spectrophotometer) tended to exceed estimations by fluorescence-based measurements (Qubit fluorometer) (**Table 2**), possibly because UV absorbance measures DNA and RNA together, while the fluorescence-based method specifically reports DNA concentration (12,13). Thus, fluorometric estimation of gDNA concentration was preferred in this study.

We compared the quality and quantity of all extracted gDNA samples. The conventional-extraction protocol maximized both concentration and DNA integrity (**Table 2, Figure 1**), making this protocol more suitable for a large-scale, high quality gDNA preparation. However, the conventional-extraction protocol demanded a great deal of hyphal material (i.e., 200-1,000 mg), more extraction steps, a larger volume of reagents, and required liquid nitrogen. Alternatively, the salt-extraction and rapid-extraction protocols can be used to extract gDNA from a smaller quantities of mycelia (i.e., 50-100 mg). Unlike with conventional-extraction, these two protocols required fewer reagents and shorter extraction time (4 hr for rapid-extract; ~6-7 hr for salt-extract; 1-2 days for conventional-extract). For smaller-scale gDNA extractions, the salt-extraction protocol was preferred because it provided higher DNA concentration (114 vs 46 ng/µl), yield (145 vs 65 ng/mg), and integrity (**Figure 1**). Moreover, the salt-extraction protocol did not require phenol, chloroform, or liquid nitrogen.

To further evaluate gDNA quality, PCR amplifications targeting three different genes (*rDNA*, *EXO1*, and *UN04715*) of *P. insidiosum* were performed, using all sets of primers (**Table 1**). Amplicon sizes ranged from ~200 to ~ 1,700 bp (**Table 1**). It was observed that impaired integrity of the gDNA generated by the rapid-extraction protocol markedly limited the success of PCR amplifications, particularly with respect to long amplicons (> 550 bp) (**Figure 2**). The gDNA templates extracted by the conventional-extraction and salt-extraction protocols may be used to amplify an amplicon size of at least 1.7 kb (**Figure 2**), indicating that these two protocols are appropriate for PCR-based applications.

In conclusion, the conventional-extraction protocol provided the highest quality and quantity of *P. insidiosum* gDNA. This protocol was suitable for the large-scale preparation of high quality gDNA. The salt-extract was a simple, rapid, and efficient protocol, making it useful for high throughput, small-scale preparation of gDNA for many molecular-biological experiments and applications. Although the rapid-extract protocol had the shortest turnaround time, the gDNA obtained was of limited quality and quantity. These observations may instruct investigators and aid their determination of which extraction protocol is optimal for a given application, dependent upon quality and quantity of gDNA desired, availability of materials and equipment, and allowable turnaround time.

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TABLES

Table 1: Primer sequences and PCR conditions for amplification of *P. insidiosum* genes (F, forward primer; R, reverse primer).

Target gene	Primer	Sequence	Amplicon size (bp)	Template amount (ng)	Anealling temperature (°C)	PCR extension time (sec)
rDNA	ITSpy1 (F) ITSpy2 (R)	5'-CTGCGGAAGGATCATTACC-3' 5'-GTCCTCGGAGTATAGATCAG-3'	233	100	60	30
rDNA	ITS1 (F) ITS4 (R)	5′-TCCGTAGGTGAACCTGCGG-3′ 5′-TCCTCCGCTTATTGATATGC-3′	931	100	55	30
EXO1	Dx3 (F) Dx4 (R)	5'-GCGAGTTCTGGCTCGACTTTA-3' 5'-ACAAGCGCCAAAAAGTCCCA-3'	550	100	57	60
UN04715	RXLR1 (F) RXLR2 (R)	5'-GCCCATGGCCTCTTCGTCCATGAGCTCGCTC-3' 5'-GCGAATTCGCACGACGGGCGGCTCGT-3'	1,575	200	50	120

Table 2: Quality and quantity of extracted genomic DNA of seven *P. insidiosum* strains. Concentrations were estimated by the UV absorbance (NanoDrop spectrophotometer) and the fluorescence-based (Qubit fluorometer) measurements. DNA yields **(**DNA obtained / mass of mycelium) were calculated based on the DNA concentration measured by the Qubit fluorometer.

Strain ID	Reference ID	Source	Concentration (ng/µl): Qubit (NanoDrop)			DNA yield (ng/mg)			A260/280 Ratio		
			Conventional extraction	Salt extraction	Rapid extraction	Conventional extraction	Salt extraction	Rapid extraction	Conventional extraction	Salt extraction	Rapid extraction
T1	CR02	Environment	336 (373)	39 (757)	79 (1,023)	67	47	136	1.9	2.0	2.0
T2	MCC18 / P12	Human (eye)	540 (574)	190 (1,590)	41 (1,330)	149	192	47	1.8	2.1	2.0
T3	Pi-S / P35	Human (leg)	271 (283)	93 (584)	43 (1,994)	122	112	55	1.8	2.1	2.1
T4	CBS777.84	Mosquito	257 (297)	86 (384)	48 (1,181)	101	146	72	1.9	2.1	2.0
T5	CBS673.85	Human (skin)	225 (351)	199 (1,652)	53 (994)	53	260	60	1.9	2.1	2.0
T6	CBS702.83	Horse	510 (557)	52 (418)	27 (360)	183	99	49	1.8	2.1	2.0
T7	P6	Human (leg)	121 (199)	139 (683)	34 (1,646)	28	162	39	1.8	2.1	2.1
Mean	-	-	323 (376)	114 (867)	46 (1,218)	100	145	65	1.8	2.1	2.0
SD	-	-	153 (141)	64 (532)	17 (520)	55	69	33	0.0	0.0	0.1

FIGURES

Figure 1: An agarose gel showing genomic DNA of *P. insidiosum* isolate T1-7, prepared by conventional-extraction, salt-extraction, and rapid-extraction protocols (M, molecular weight markers).

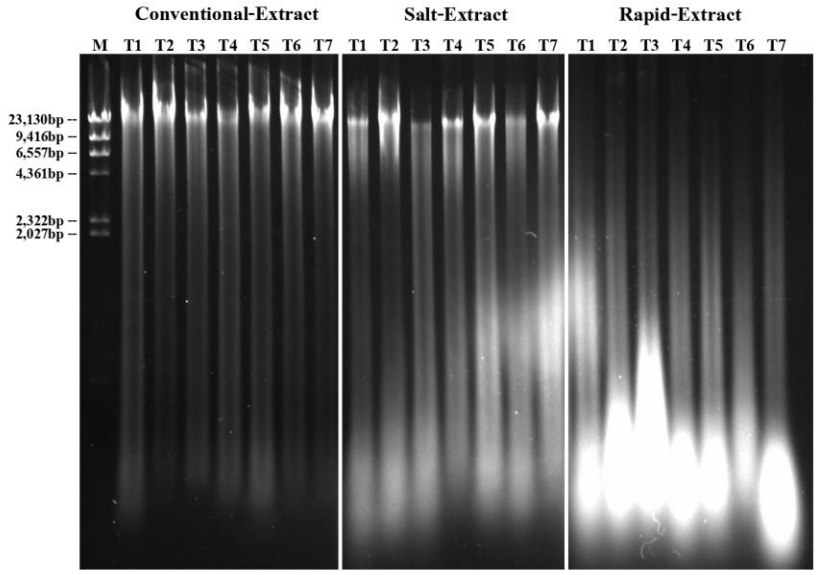
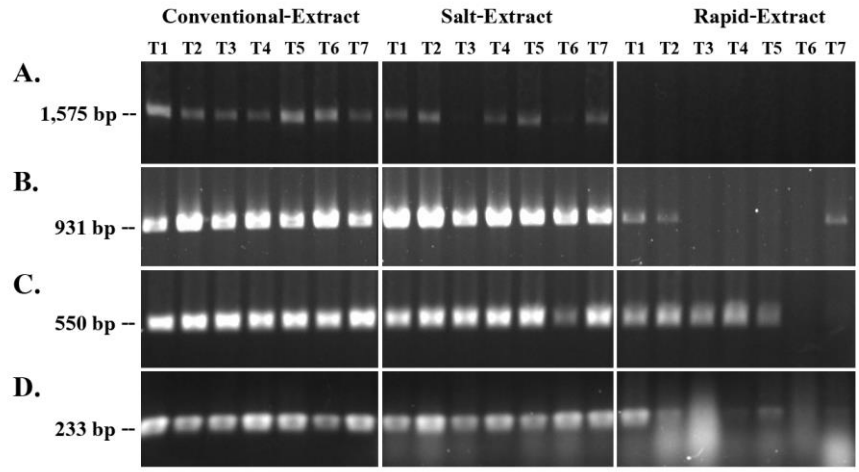


Figure 2: PCR amplification of *P. insidiosum* genes using the DNA templates prepared by conventional-extraction, the salt-extraction, and the rapid-extraction protocols: **A**. the putative RXLR-effector encoding gene (*UN04715*) amplified by the primers RXLR1 and RXLR2; **B**. the ribosomal DNA gene (*rDNA*) amplified by the primers ITS1 and ITS4; **C**. the putative exo-1,3-beta glucanase-encoding gene (*EXO1*) amplified by the primers Dx3 and Dx4; and **D**. *rDNA* amplified by the primers ITSpy1 and ITSpy2.



TITLE: Transcriptome Analysis Reveals Pathogenicity and Evolutionary History of the Pathogenic Oomycete *Pythium insidiosum*

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SHORT TITLE: Transcriptome of *P. insidiosum*

KEY WORDS: Pythiosis, *Pythium insidiosum*, Oomycete, Transcriptome, Expressed sequence tag

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ABSTRACT

Oomycetes form a unique group of microorganisms that share hyphal morphology with fungi. Most of pathogenic oomycetes infect plants, while some species are capable of infecting animals. *Pythium insidiosum* is the only oomycete that can infect both humans and animals, and causes a life-threatening infectious disease, called "pythiosis". Controlling an infection caused by *P. insidiosum* is problematic because effective antimicrobial drugs are not available. Information on the biology and pathogenesis of *P. insidiosum* is limited. We generated a *P. insidiosum* transcriptome of 26,735 unigenes, using the 454 sequencing platform. As adaptations to increased temperature inside human hosts are required for a successful pathogen, we generated *P. insidiosum* transcriptomes at 28˚C and 37˚C and identified 625 up-regulated and 449 down-regulated genes at 37˚C. Comparing the proteomes of oomycetes, fungi, and parasites provided clues on the evolutionary history of *P. insidiosum*. Potential virulence factors of *P. insidiosum*, including putative RXLR and elicitin effectors, were indentified. *P. insidiosum* harbored an extensive repertoire of ~300 elicitins that is at least 5 times more than that found in other oomycetes. Fifteen putative RXLR translocation effectors were identified in *P. insidiosum*, whereas none are found in the closely-related oomycete *Pythium ultimum*. Evolution, through gene gain, gene loss, and gene modification has produced a wide variety of effectors in oomycetes. Such evolutionary events are likely required for the host specificity and virulence strategies of a particular oomycete species. The transcriptome, presented herein, is an invaluable resource for exploring *P. insidiosum*'s biology, pathogenesis, and evolution.

1. INTRODUCTION

Members of the genera *Pythium*, *Albugo*, *Peronospora*, *Hyaloperonospora, Phytophthora*, *Plasmopara*, *Bremia*, *Aphanomyces*, *Lagenidium* and *Saprolegnia* form a unique group of microorganisms, called "oomycetes", which belong to the Stramenopiles of the supergroup Chromalveolates (Kamoun 2003; Keeling *et al.* 2005). Although oomycetes share hyphal morphology with the fungi, molecular phylogenetic analysis shows that they are more closely related to diatoms and algae (Kwon-Chung 1994). Most of the pathogenic oomycetes infect plants, while a few species of the genera *Pythium*, *Aphanomyces*, *Lagenidium*, and *Saprolegnia* are capable of infecting humans or animals (Kamoun 2003). Controlling infections caused by the oomycetes is problematic because effective antimicrobial drugs are not available.

P. insidiosum is the only oomycete that infects both humans and animals, and causes a life-threatening infectious disease, called "pythiosis". The mechanisms that *P. insidiosum* has evolved to be a successful human pathogen remains unknown. *P. insidiosum* inhabits swampy areas, where it is present as mycelia colonizing the surface of water plants. Similar to other oomycetes, *P. insidiosum* generates motile zoospores (Mendoza *et al.* 1993). When a zoospore comes in contact with a human or animal, it can geminate as hyphae and invade host tissues. Patients with pythiosis usually present with clinical symptoms relating to a progressive infection of arterial, ocular, cutaneous/subcutaneous, or gastrointestinal tissues (Mendoza *et al.* 1996; Krajaejun *et al.* 2006). Pythiosis is notorious for high rates of morbidity and mortality (Mendoza *et al.* 1996; Krajaejun *et al.* 2006). Conventional antifungal drugs are mostly unsuccessful for treatment of pythiosis. Extensive surgery of infected tissues is the main option for treatment of pythiosis, but it leads to life-long disabilities for most patients, and many die from advanced infections (Mendoza *et al.* 1996; Krajaejun *et al.* 2006). Immunotherapy using a crude extract prepared from *P.*

insidiosum culture has limited efficacy for the treatment of pythiosis (Mendoza and Newton 2005; Krajaejun *et al.* 2006). Thus, the discovery of more effective treatments of the disease is an urgent and important healthcare goal.

Knowledge of the biology, evolution, and pathogenesis of *P. insidiosum* is limited and is hampered by the lack of molecular information from the genome. While genome sequences of many pathogenic oomycetes are publicly available (Tyler *et al.* 2006; Tyler 2009; Haas *et al.* 2009; Levesque *et al.* 2010; Baxter *et al.* 2010; Links *et al.* 2011; Lamour *et al.* 2012), the *P. insidiosum* genome has not been sequenced. As an alternative to complicated whole genome sequencing, transcriptome analysis is a faster and cost-effective way to explore the biology and virulence components of many related oomycetes (Kamoun *et al.* 1999; Qutob *et al.* 2000; Torto-Alalibo *et al.* 2005; Randall *et al.* 2005; Gaulin *et al.* 2008; Le Berre *et al.* 2008). Recently, a set of ~200 *P. insdisiosum* genes from a cDNA library were identified, using the Sanger chain-termination sequencing method (Krajaejun *et al.* 2011). However, this dataset is too small, and cannot demonstrate a comprehensive view of *P. insidiosum* biology.

In this study, we report on a large-scale transcriptome analyasis of *P. insidiosum*, using the 454 sequencing platform. The ability to survive at body temperature in mammalian hosts is a crucial factor for successful pathogens. We compared transcriptome profiles of *P. insidiosum* growing at body and room temperatures, in order to identify up- and down-regulated genes in response to a thermal stress. Genetic comparison of the *P. insidiosum* to other oomycetes, fungi, and parasites, could provide clues for the pathogenesis- and evolution-related questions of why and how *P. insidiosum* is the only oomycete that infects humans. Identification of *P. insidiosum* virulence factors orthologous to that of other microorganisms could suggest the presence of shared pathogenesis mechanisms among the pathogens. Therefore, the transcriptome analysis reported here is a significant step for the study of *P. insidiosum*, and it could lead to the identification of novel drug targets for the discovery of new treatment strategies for pythiosis.

2. MATERIALS AND METHODS

2.1 Strain, growth condition, and RNA extraction

The *P. insidiosum* strain Pi-S, 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 two different temperatures, 28 °C (room temperature; RT) and 37 °C (body temperature; BT), for one week. Mycelia, from each growth condition, was rinsed briefly with pre-warmed (28 or 37 °C) sterile water. The mycelia was transferred to a 2-ml microcentrifuge tube (~500 mg of hyphae/tube), and re-incubated at previous growth temperatures for 24 hours. Growth conditions were identical, except for temperature. Microcentrifuge tubes containing mycelia were then snap-frozen in liquid nitrogen, and stored at -80 °C until use. The frozen hyphae were disrupted two times with glass beads (diameter, 710-1,180 µm, Sigma), using TissueLyzer MM301 (Qiagen, Germany), with the following setting: 2 min at 30 Hz. Total RNA was extracted using Trizol Reagents (Invitrogen, Carlsbad, CA), and then purified using an RNeasy Mini kit (Qiagen), according to the manufacturers' protocols. The obtained total RNA was treated with DNAfree Turbo (Ambion) to remove contaminating DNA. The total RNA concentration and purity were measured using spectrophotometry (NanoDrop 2000, Thermo scientific), and the integrity was evaluated by agarose gel electrophoresis. The total RNA was stored at -80 °C until use for the mRNA purification step.

2.2 cDNA library construction and 454 pyrosequencing

A total of 200 ng of poly-A mRNA sample was isolated from the total RNA, using an Absolutely mRNA Purification kit (Stratagene), and fragmented in 1x fragmentation buffer (0.1 M $ZnCl_2$, 0.1 M Tris-HCl, pH7.0) at 70 °C for 30 sec. The reaction was stopped by adding 2 µl of 0.5 M EDTA and 28 µl of 10 mM Tris HCl, pH7.5. The mRNA sample was cleaned using Agencourt RNAClean reagent (Beckman Coulter), washed with 200 µl of 70% EtOH, air dried and eluted in 20 µl of 10 mM Tris HCl, pH7.5. Fragmented mRNA samples were separated by RNA 6000 Pico kit on the Agilent 2100 bioanalyzer (Agilent Technologies). Fragmented mRNA samples were converted to double-stranded cDNA with the cDNA Synthesis System Kit (Roche Applied Science) using random primers and AMV Reverse Transcriptase. A cDNA library for 454 pyrosequencing was prepared according to the cDNA Rapid Library Preparation protocol (Roche Applied Sciences). The cDNA library was amplified in emulsion PCR and subject to pyrosequencing on a picotitre plate of the Genome Sequencer (GS) FLX Titanium platform (Roche Applied Sciences).

2.3 Sequence assembly

Assembly of 454 GS-FLX sequences was performed using iAssembler (Zheng *et al.* 2011). FASTA and associated quality files were firstly preprocessed for the removal of sequence adapters. Cleaned files were passed to iAssembler to obtain the unique sequences (unigenes). iAssembler is a standalone Perl package, which assemble ESTs using repetitive cycles of MIRA, followed by CAP3 assembly.

2.4 Annotation and functional assignments

All unigenes were conceptually translated into putative proteins, using the ESTscan program (Iseli *et al.* 1999). All the unigenes and predicted proteins were annotated using several bioinformatic tools. First, the protein sequences were searched for significant similarities (E-value cut-off $\leq 1e^{-4}$), utilizing the BLASTP algorithm and the NCBI's non-redundant protein database. Second, InterProScan (Zdobnov and Apweiler 2001), NCBI conserved domain search (Marchler-Bauer *et al.* 2011), and BLAST2GO (Conesa *et al.* 2005) assisted identification of protein domains and Gene Ontology (GO) of annotated proteins. Third, mapping the *P. insidiosum* proteins to the KEGG pathways (Kanehisa and Goto 2000) was done by using the KAAS program (Moriya *et al.* 2007).

2.5 Comparison of *P. insidiosum* transcriptome datasets

All unigenes of the combined BT-RT dataset were assigned the unique gene ID numbers, and were used as the reference genes for comparison of the *P. insidiosum* transcripts differentially expressed at body and room temperatures. The reference gene dataset was formatted to make a BLAST database using formatdb. BLAST was run against each of the BT and RT datasets separately, with all single reads (the unassembled FASTA). BLAST results were parsed using Perl and UNIX shell utilities, in the format required, to obtain counts of each transcript for each growth condition against the reference genes. Multidiff (Herbert *et al.* 2008) was then run to carry out differential expression analysis.

2.6 Comparison of oomycete, fungal, and parasitic proteome

SimiTri (Parkinson and Blaxter 2003) was used to compare the predicted *P. insidiosum* proteins to a set of three proteomes: (i) pathogenic oomycetes (*Pythium ultimum, Phytophthora sojae, Saprolegnia parasitica*), (ii) pathogenic fungi (*Aspergillus flumigatus, Cryptococcus neoformans* var. *grubii* [strain H99]*, Histoplasma capsulatum* [strain G186AR]), and (iii) parasites (*Plasmodium falciparum* [strain Dd2], *Brugia malayi*, *Caenorhabditis elegans*).

Protein sequences of these organisms were retrieved from UniProt ('Reorganizing the protein space at the Universal Protein Resource (UniProt)' 2012) (for *B. malayi* and *C. elegans*), http://pythium.plantbiology.msu.edu/ (for *P. ultimum*), and http://www.broadinstitute.org/ (for the rest).

2.7 Identification of putative virulence factors of *P. insidiosum*

P. insidiosum proteins were used to blast against the MvirDB database, an updated collection of known virulence factors of a variety of pathogens (Zhou *et al.* 2007). To set up a local sequence homology searching tool, the NCBI-BLAST 2.2.25+ was downloaded from http://www.ncbi.nlm.nih.gov/, and installed, according to the program instructions. The *P. insidiosum* unigenes and proteins (from the BT-RT combined datasets) were formatted to make a BLAST database, which facilitated BLAST searching of virulence factors, effectors, and biologically-important genes.

Effectors (i.e., crinkler, RXLR, and elicitin proteins) are a group of secreted molecules that facilitate infection processes of many plant pathogens, including oomycetes (Kamoun 2006; Levesque *et al.* 2010). Signal peptide (by SignalP 3.0; HMM score > 0.9) (Bendtsen *et al.* 2004), and effector-specific domains or motifs (by sequence homology search and InterProScan analyses) were used to identify *P. insidiosum* effectors. Crinklers contain a signal peptide, the N-terminal conserved LFLAK domain (containing the "LXLFLAK" motif for *Phytophthora* species, and "LXLYLAR/K" for *Pythium ultimum*), and the DWL domain (containing the "HVLVXXP" motif) (Torto *et al.* 2003; Haas *et al.* 2009; Levesque *et al.* 2010). Sequences of 50 crinkler effectors of three *Phytophthora* species (Haas *et al.* 2009), and two prototypic *P. infestans* crinkler effectors [CRN1 (NCBI accession: AAN31500.1) and CRN2 (AAN31502.1)] (Torto *et al.* 2003) were used to BLAST search against all 24,610 predicted proteins of *P. insidiosum*.

RXLR effectors contain a signal peptide, the N-terminus "RXLR-dEER" domain (containing RXLR and dEER motifs, which are necessary for translocation of the effector to host cell), and the C-terminus functional domain (necessary for pathogenicity) (Kamoun 2006; Morgan and Kamoun 2007; Govers and Bouwmeester 2008). "RXLR" string search was used to identify putative RXLR effectors among the secreted proteome of *P. insidiosum*. The obtained candidates were then manually checked for the presence of the RXLR motif located near the predicted signal peptide cleavage site, as well as, the presence of the dEER motif (a stretch of mainly acidic amino acids, i.e., D and E) located right after the RXLR motif.

Elicitins are a large protein family with the conserved elicitin domain (~98 amino acids long with six cysteine residues) (Yu 1995; Panabières *et al.* 1997; Jiang *et al.* 2006). Interproscan (Zdobnov and Apweiler 2001) and NCBI conserved domain searches (Marchler-Bauer *et al.* 2011) were used to identify putative elicitin proteins of *P. insidiosum*. Online bioinformatic programs were used to predict signal peptides (SignalP3.0 (Bendtsen *et al.* 2004)), N-glycosylations (www.cbs.dtu.dk/services/NetNGlyc), O-glycosylations (www.cbs.dtu.dk/services/NetOGlyc) (Julenius *et al.* 2005), and glycosylphosphatidylinositol (GPI) anchors (Eisenhaber *et al.* 2004) for putative elicitin proteins.

2.8 Phylogenetic analysis

The analysis was performed on the Phylogeny.fr platform (Dereeper *et al.* 2008). First, sequences were aligned with MUSCLE (v3.7), configured for highest accuracy (Edgar 2004). After alignment, ambiguous regions (gaps or poorly aligned) were removed with Gblocks (v0.91b) (Castresana 2000). The phylogenetic tree was

reconstructed using the maximum-likelihood method implemented in the PhyML program (v3.0), and the reliability of internal branches was assessed using the aLRT test (Guindon and Gascuel 2003; Anisimova and Gascuel 2006). The graphic representation of the phylogenetic tree was produced with Dendroscope (v3.2.2) (Huson *et al.* 2007).

2.9 Nucleotide sequence accession

All 26,735 unigenes of *P. insidiosum* derived in this study, UN00001-UN26735, have been submitted to the DNA Data Bank of Japan under accession numbers FX526991 to FX553725, respectively.

3. RESULTS

3.1 Transcriptome profile of *P. insidiosum*

Two transcriptome datasets of the *P. insidiosum* clinical strain Pi-S, growing at two different temperatures (BT or 37°C; and RT or 28°C), were generated by 454 pyrosequencing. The sequencing outputs (i.e., raw bases, raw reads, and average read length) of the two datasets were similar (**Table 1**). When all the raw data was combined into a BT-RT dataset there were ~400,000 reads, comprising ~200 million bases, with an average read length of ~500 bases (**Table 1**).

To generate a collection of unique sequences (unigenes), raw reads of each dataset were assembled using iAssembler (Zheng *et al.* 2011). The total bases of assembled BT and RT datasets were reduced by 7.4 and 7.8 fold, respectively. Profiles of the BT and RT datasets (i.e., number, average length, and CG content of unigenes) were very similar (**Table 1**). After assembly, the combined BT-RT dataset contained ~27,000 unigenes, covering ~20 million bases (10-fold reduction of raw bases), and an average gene length of ~760 bases (**Table 1**). Based on ESTscan analyses (Iseli *et al.* 1999), at least 92% of the unigenes in the BT, RT, and combined BT-RT datasets can be translated into putative proteins.

3.2 Annotation of *P. insidiosum* transcriptome

For the combined BT-RT dataset, ~80% of the *P. insidiosum* proteins matched with a protein in the NCBI database (**Table 1 and S1**). A substantial proportion (~30%) of the *P. insidiosum* annotated proteins fell into a group of conserved hypothetical proteins with unassigned function. Of the functionally-assigned proteins, the ribosomal proteins formed a notable group, which accounted for ~5% of all predicted proteins.

Features and classifications of the *P. insidiosum* proteome were determined by KEGG pathway assignment, gene ontology (GO) grouping, and protein domain searchs. The KAAS web-based interface assigned the K-numbers (each K-number represents a biological role) to *P. insidiosum* proteins, based on similarity to KEGG-defined proteins of the reference organism *Saccharomyces cerevisiae* (Kanehisa and Goto 2000; Moriya *et al.* 2007). A total of 5,332 proteins of *P. insidiosum* (22% of all predicted proteins) matched 1,320 K-numbers in 254 KEGG pathways. Major (parent) KEGG pathways, found in *P. insidiosum*, can be divided into 4 groups: (i) Metabolism, (ii) Genetic information processing, (iii) Environmental information processing, and (iv) Cellular processes **(Table 2)**. GO terms were extracted from the annotated proteins of the combined BT-RT dataset by using BLAST2GO (Conesa *et al.* 2005). The GO terms were categorized into three main groups, including cellular components, biological processes, and molecular functions, as depicted in **Figure 1**. Interproscan (Zdobnov and Apweiler 2001) mapped functional domains to predicted proteins. A total of 3,558 domains were identified in 12,118 proteins of *P. insidiosum*, and the top 30 protein domains are shown in **Table 3**.

3.3 Thermally up- and down-regulated genes of *P. insidiosum*

All unassembled raw reads of the RT and BT datasets were matched against all 26,735 unigenes in the reference BLAST dataset to obtain counts of each transcript. The Multidiff program (Herbert *et al.* 2008), with a potent cut-off stringency (Q-value < 0.05), was used to analyze differential expression of each gene. A total of 1,074 genes of *P. insidiosum* growing at body temperature, were significantly up-regulated (625 genes) or down-regulated (449 genes), in comparison with the growth at room temperature **(Table S2)**. Based on the BLAST search results, these 1,074 genes can be divided into 309 groups (up to 408 genes per group). The top 40 groups (based on number of genes) were listed in **Table 4**.

3.4 Proteome comparison of oomycetes, fungi, and parasites

By using the SimiTri program (Parkinson and Blaxter 2003), the *P. insidiosum* proteome (24,610 predicted proteins) were compared to a set of three proteomes from other microorganisms: (i) oomycetes [*P. ultimum* (12,614 proteins)*, P. sojae* (16,989 proteins)*, S. parasitica* (20,088 proteins)], (ii) fungi [*A. flumigatus* (9,887 proteins)*, C. neoformans* var. *grubii* (6,967 proteins), *H. capsulatum* (9,233 proteins)], and (iii) parasites [*P. falciparum* (5,139 proteins), *B. malayi* (11,565 proteins), *C. elegans* (25,547 proteins)]. Approximately, 70% (17,171 proteins), 34% (8,357 proteins), and 34% (8,341 proteins) of the *P. insidiosum* proteins were similar to the proteins of at least one species in the oomycete, parasitic, and fungal sets, respectively (**Figure 2A-C**). The *P. insidiosum* proteins were also compared to the proteome of a representative organism from each set. The SimiTri analysis showed that 64%, 17%, and 32% of the *P. insidiosum* proteins shares sequence similarity to the proteins of the oomycete *P. sojae*, the parasite *P. falciparum*, and the fungus *A. flumigatus*, respectively. Overall, only 3,815 proteins (16%) of *P. insidiosum* were shared among all three representative pathogens, but were more closely related to the oomycete organism (**Figure 2D**).

3.5 Putative virulence factors of *P. insidiosum*

The MvirDB database is a comprehensive collection of virulence factors from a variety of human pathogens (Zhou *et al.* 2007). So far, there are 29,576 records, or MvirDB-defined virulence factor identifications (VFIDs), that can be categorized into six groups: virulence proteins (n=8,726), protein toxins (n=8,632), antibiotic resistances (n=5,642), pathogenicity islands (n=5,895), transcription factors (n=557), and others (n=124). All 24,610 predicted proteins of *P. insidiosum* were BLAST searched against the MvirDB databases and 1,974 *P. insidiosum* proteins matched 488 VFIDs **(Table S3)**. The top 40 VFIDs with the highest number of *P. insidiosum* proteins are shown in **Table 5**. Of the 625 *P. insidiosum* proteins which were up-regulated at 37 °C, 85 matched VFIDs. The top 35 VFID-matched and highly up-regulated proteins of *P. insidiosum* are shown in **Table 6**.

3.6 Putative *P. insidiosum* effectors

3.6.1 Crinklers: Based on sequence homology analysis, only three *P. insidiosum* proteins significantly matched (E-value $\leq 1e^{-4}$) the crinklers of *Phytophthora* species. One of these *Phytophthora* homologs matched the *P. infestans* crinkler CRN2 (with an E-value = 2e-012). These *P. insidiosum* proteins had the LFLAK motif (containing "LQLYLAK", "LKLYLAR" or "LDLGYLAR") and the DWL motif (containing "HVLVXXP" or "NVLVXXP"). However, none of them had a predicted signal peptide (by SignalP3.0 analysis).

3.6.2 RXLR effectors: The "RXLR" search against the secreted proteome of *P. insidiosum* identified 15 proteins

with the RXLR motif resided within 60 amino acids (average distance, 37 amino acids; range, 3-60) from the signal peptide cleavage site (**Table 7**). The dEER motif search revealed up to seven acidic amino acids located within the 30 amino acids downstream of the RXLR motif in all 15 *P. insidiosum* proteins (**Figure 3**). Twelve putative RXLR effectors matched four hypothetical and eight annotated proteins in the NCBI database (**Table 7**). Eleven putative *P. insidiosum* RXLR effectors matched proteins of other oomycetes: *P. infestans* (n=10) and *Albugo laibachii* (n=1). One *P. insidiosum* protein hit a reported RXLR effector of *P. infestans* (E-value = 3E-80).

3.6.3 Elicitins: Interproscan (Zdobnov and Apweiler 2001) and NCBI conserved domain searches (Marchler-Bauer *et al.* 2011) co-identified an elicitin domain in 294 *P. insidiosum* proteins (average length, 258 amino acids; range, 52-1,247). Of these proteins, 226 had only one (n=214) or two (n=12) elicitin domains, while 68 possessed an elicitin domain and additional functional domains (**Figure 4B**). Sixty one of the *P. insidiosum* elicitin-like proteins were predicted to be secreted by SignalP, and 44, 20 and three of them harbored N-glycosylation sites, O-glycosylation sites, and GPI anchors, respectively. Two hundreds and eighteen proteins had full-length elicitin domains (average length, 83 amino acids; range, 56-92), which can be grouped into 98 unique elicitin domain sequences. Alignment of the 98 unique full-length elicitin domain sequences of *P. insidiosum* showed a high degree of similarity (**Figure 4A**). A reconstructed phylogenetic tree (based on the maximum likelihood method) of the 98 elicitin domain sequences of *P. insidiosum* and 38 representative elicitin domain sequences of other oomycetes (Jiang *et al.* 2006) demonstrated 10 clades: four of which (clade A, B, C, D) contained multiple sequences, while the other clades (clade E, F, G, H, I, J) contained only one sequence (**Figure 5**). Only the branch support values of at least 70% (calculated by the approximate likelihood-ratio test) were shown at corresponding nodes (Anisimova and Gascuel 2006).

4. DISCUSSION

The majority (~80%) of *P. insidiosum* proteins were orthologous to the proteins of other organisms (**Table 1 and S1**). Strikingly, almost all BLASTP-matched proteins of *P. insidiosum* (~90%) were homologous to proteins of other oomycetes, confirming the close evolutionary relatedness of the oomycetes (Kwon-Chung 1994; Latijnhouwers *et al.* 2003). Nevertheless, KEGG analysis (Kanehisa and Goto 2000; Moriya *et al.* 2007) of *P. insidiosum* proteins indicated many shared metabolic pathways with the model eukaryotic organism *S. cerevisiae* (**Table 2**). Components of *S. cerevisiae* pathways, involved in metabolism of carbohydrates, nucleotides, cofactors and vitamins, and some other cellular processes, were also found in *P. insidiosum*, suggesting that, although remarkably diverged, these pathways are conserved and necessary among these eukaryotes. However, in several major (parent) metabolic pathways, more than half of the subset (daughter) pathways were lacking in *P. insidiosum*, e.g., metabolism of glycan, terpenoids, polyketides, and other secondary metabolites (**Table 2**).

Among the identified protein domains (**Table 3)**, protein kinases were the most abundant domain found in *P insidiosum* (1,081 hits). Similar to other eukaryotes, the protein kinases seemed to be crucial in *P. insidiosum* because phosphorylation is a main biochemical process that is involved in many cellular activities (Hanks 2003). Some other prominent protein domains discovered in *P. insidiosum* included elicitin (303 hits), major facilitator (MFS) and ATP-binding cassette transporter (ABC) superfamilies (276 hits), WD40-repeat (240 hits), ankyrin repeat

(231 hits), EF-hand (226 hits), and armadillo-type fold (222 hits) (**Table 3**). All of these domains, with an exception of elicitin, are found in many different types of proteins that participate in major intracellular activities of almost, if not all, eukaryotes, including oomycetes.

The proteome of *P. insidiosum* was compared to that of plant-pathogenic oomycetes, pathogenic fungi, and some parasites (**Figure 2**). As expected for the oomycete group, a majority (~17,200) of *P. insidiosum* proteins shared with at least one of the three oomycete species tested, while the rest (~7,400) were unique to *P. insidiosum* (**Figure 2A**). In contrast, markedly fewer proteins of *P. insidiosum* shared sequence similarity to that of the parasites (~8,400 proteins; **Figure 2B**) and the fungi (~8,300 proteins; **Figure 2C**). Moreover, the proteome of *P. insidiosum*, when compared to members of different groups of eukaryotes, showed more homologies to the oomycetes than the parasites or fungi (**Figure 2D**). These observations supported the unique evolution, physiological life style, and perhaps pathogenesis strategy of the oomycetes, in relation to those of fungi and parasites.

The temperature optimum for growth of most *Pythium* species is less than 30 °C, while just a few species are able to grow well at higher temperatures (van der Plaats-Niterink 1981). Thermal tolerance at body temperature (37 °C) is required for pathogenesis of successful human pathogens, and is therefore considered as a virulence factor (Steen *et al.* 2002). Transcripts of *P. insidiosum* growing at room (28 °C) and body temperatures were compared. A total of 1,074 transcripts, which can be divided into 309 gene product groups, were significantly up (625 genes) or down (449 genes) regulated at 37 °C. Forty-five percent of these transcripts matched gene products with unknown function (Group1) or had no BLAST hit (Group2) (**Table 4**). The remaining 55% of the transcripts were functionally annotated (**Table 4**). For example, 37 of 50 (74%) ribosomal proteins (Group3) and eight of nine (89%) translation elongation factors (Group17) were down-regulated at 37 °C. This suggests that there were changes in protein synthesis in *P. insidiosum* adjusting to body temperature. Proteolytic activity of proteases has been thought to be a necessary process for tissue invasion of *P. insidiosum*, because of insufficient hyphal exertion force (Ravishankar *et al.* 2001). Production of proteases has been reported previously in *P. insidiosum* (Davis *et al.* 2006). In the *P. insidiosum* transcriptome, we found 15 of 19 proteolytic enzymes, including serine- (Group11), cysteine- (Group12), aspartyl- (Group19), and metallo- (Group40) proteases, were markedly up-regulated at 37 °C (**Table 4**). The presence of these thermally-regulated proteases supports the idea of the synergy of hyphal exertion force and proteolysis in the penetration of mammalian tissue. Regarding elicitins, expression of 20 elicitins (Group6) were altered when the pathogen was growing at 37 °C, and among them, 17 elicitins (85%) were up-regulated (**Table 4**). Thus the *P. insidiosum* elicitins form an interesting group of proteins that are abundant in *P. insidiosum*, are not present in other human pathogens, and are, for many of them, up-regulated at body temperature, suggesting an important role in pathogenesis. Taken together, the changes in the transcriptome associated with growth temperature indicates a set of genes that may be critical for promoting pathogenicity inside mammalian hosts.

For *P. insidiosum*, the intersection of the set of thermally up-regulated proteins, and the set of MvirDB-matched proteins, includes a confined set of 85 proteins, 35 of which had very strong differential gene expression scores (Q-value < 0.01) (**Table 6**). Since *P. insidiosum* is the sole human pathogen among the oomycetes, another gene sets to consider for investigation of virulence factors would include those that are unique to *P. insidiosum* and

not found in other oomycetes. There were ~7,400 proteins of *P. insidiosum* that were not orthologous to any proteins of the closely-relate *Pythium* species (*P. ultimum*), the animal-pathogenic oomycete *S. parasitica*, and the plant-pathogenic oomycete *P. sojae* (**Figure 2**). However, among these *P. insidiosum*-specific proteins, none was both thermally up-regulated and MvirDB-matched.

Effectors are secreted proteins that facilitate the infection process of many pathogens (Kamoun 2006). Some effectors interact with the host extracellularly, whereas others can translocate and modulate host cell functions intracellularly. In plant-pathogenic oomycetes, RXLRs and crinklers are among the large intracellular effector families that have been well studied so far. However, the existence of these effectors in human- and animal-pathogenic oomycetes, including *P. insidiosum*, was previously lacking. Crinklers require the conserved N-terminus LFLAK and DWL domains for translocation into host cells, and also need the variable C-terminal portion for targeting the nucleus and inducing cell death (Haas *et al.* 2009; Schornack *et al.* 2010; van Damme *et al.* 2012). No typical crinkler molecule was identified in *P. insidiosum*, in contrast, there are up to several hundred crinkler effectors in other oomycetes (**Table 8**). For RXLRs, these effectors use the RXLR-dEER domain to get inside host cell, and then use a functional C-terminual domain to alter host cell responses (Morgan and Kamoun 2007; Dou *et al.* 2008a, b; Govers and Bouwmeester 2008; Tyler 2009). The translocation function of the RXLR-dEER domain is similar to that of the PEXEL domain (containing the RXLX motif) of the effectors from the malarial parasite *Plasmodium falciparum* (Hiller *et al.* 2004; Marti *et al.* 2004). These PEXEL and RXLR-dEER translocation domains are functionally interchangeable (Morgan and Kamoun 2007; Govers and Bouwmeester 2008). *Phytophthora* and *Hyaloperonospora* species have large repertoires of the RXLRs, while some other oomycetes possess none or a few typical RXLR molecules (**Table 8**). These results indicated that, during evolution, the RXLR effectors were extensively expanded in Peronosporales compared to other oomycete lineages (Beakes *et al.* 2012). In contrast to *P. ultimum*, which has no RXLRs, 15 putative RXLR effectors were found in *P. insidiosum* (**Table 7**). Structurally, RXLR domains of the putative RXLR proteins of *P. insidiosum* were similar to that of the *P. falciparum* PEXEL and other oomycetes RXLR effectors (**Figure 3**). Further functional characterization is needed to elucidate if any of these putative *P. insidiosum* RXLR effectors have such a translocation role that mediates virulence in humans, as observed in the PEXEL effectors of *P. falciparum*.

A striking observation about the *P. insidiosum* effectors was the discovery of an extensive repertoire of elicitins, which are oomycete-specific proteins (Jiang *et al.* 2006). In other oomycetes studied to date, the number of elicitins ranges from one in *A. euteiches* to 57 in *P. sojae* (**Table 8**). In contrast, *P. insidiosum* harbored 294 elicitins with five different modular protein organizations (**Figure 4B**). Elicitins can trigger defense responses and programmed cell death of host plant cells, and promote infection (Jiang *et al.* 2006). They are also recognized as a pathogen-associated molecular pattern (PAMP) in plants (Nürnberger *et al.* 2004). Elicitins can also act as sterol-carrying proteins, in order to acquire exogenous sterols for growth of oomycetes (i.e., *Phytophthora* and *Pythium* species), which lack the endogenous ergosterol biosynthesis pathway (Boissy *et al.* 1999; Madoui *et al.* 2009; Gaulin *et al.* 2010). Unique elicitin domain sequences from *P. insidiosum* (n=98) and *Phytophthora* and other *Pythium* species (n=38) can be phylogenetically divided into 10 distinct clades (**Figure 5**). The clade-A contained only *P. insidiosum* elicitins. Many *P. insidiosum* elicitins are predicted to be secreted (presence of signal peptide) or cell-wall associated (presence of GPI anchor or glycosylation site) (Jiang *et al.* 2006), making each elicitin a good

candidate for being a specific diagnostic marker or a candidate for the development of an anti-oomycete molecule, e.g., antibodies, vaccines and drugs. Further study is needed to demonstrate the biochemical and pathogenicity properties of the *P. insidiosum* elicitins in human hosts.

In conclusion, the transcriptome database described here forms an invaluable resource for exploring the biology, pathogenesis, and evolution of *P. insidiosum*. Many putative virulence proteins were identified, and they provide clues on pathogenic mechanisms of *P. insidiosum*. Evolution, through gene gain, gene loss, and gene modification has produced a wide variety of effectors in oomycetes. Such evolutionary events are likely required for the specialization of lifestyle, host specificity, and virulence strategies of a particular oomycete species. From a medical perspective, many of the predicted *P. insidiosum* proteins, including putative virulence factors and effectors, should be useful as targets for the development of a better diagnostic and treatment modalities for pythiosis.

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6. FIGURE LEGENDS

Figure 1. Gene ontology (GO) of *P. insidiosum* assigned by BLAST2GO: **A**, Cellular component; **B**, Biological process; **C**, Molecular function.

Figure 2. Phylogenetic relationship of three groups of eukaryotic microorganisms: By using the SimiTri analysis platform, the *P. insidiosum* proteins (n = 24,610) were compared to the proteome of other microorganisms: (**A)** oomycetes [*P. ultimum*, *P. sojae*, and *S. parasitica*], (**B**) parasites [*P. falciparum*, *B. malayi*, *C. elegans*], and (**C**) fungi [*A. flumigatus, C. neoformans* var. *grubii*, *H. capsulatum*]. The *P. insidiosum* proteome were also compared to the proteome of a representative organism from each group (*P. sojae*, *P. falciparum*, and *A. flumigatus*) (**D**). The numbers at each vertex represent the number of proteins matching only that specific database. The numbers on the edges represent the number of proteins matching the two databases linked by that edge. The numbers within the triangle represent the number of *P. insidiosum* proteins with matches to all three databases. The numbers outside the triangle in a box represent the number of *P. insidiosum* proteins specific to *P. insidiosum* during the comparison.

Figure 3. Amino acid alignment of the RXLR/RXLX effectors: The RXLR translocation domains of all 15 putative RXLR effectors of *P. insidiosum* (**Table 8**) were compared to that of the well-studied RXLR/RXLX effectors of other oomycetes (Avr1b, Avr3a, ATR1 and ATR13), and the malarial parasite *P. falciparum* (EMP3, HRPI, HRPII, and GBP130). Signal peptides were excluded from the alignment. The first amino acid of each sequence was the

predicted signal peptide cleavage site. RXLR or RXLX motifs are boxed; acidic amino acids of the dEER motifs are underlined.

Figure 4. Sequence alignment and domain organization of the elicitin proteins: (**A**) Alignment of the 218 full-length elicitin domain sequences, using the Clustal Omega, showed a high degree of sequence conservation (upper bar graph). Alignment quality of each amino acid residue (depends on numbers and types of the amino acid at that alignment position) was demonstrated in the middle bar graph. The consensus sequence was presented in the lower bar graph. Six cysteine residues (a characteristic of elicitin domain) were indicated by asterisks. (**B**) Diagrams showed five different domain organizations of 294 *P. insidiosum* elicitin-like proteins (Other, other functional domains; Elicitin, elicitin-like domain).

Figure 5. Phylogenetic analysis of oomycete elicitins: The maximum likelihood method was used to generate a phylogenetic tree of the 134 unique full-length elicitin domain sequences from *P. insidiosum* (n=98; INSL001-098) and other oomycetes (n=36; indicated by asterisks). The tree showed four major (multiple sequences per clade; as indicated by A, B, C and D) and six minor (one sequence per clade; as indicated by E, F, G, H, I and J) clades. Only the branch support values of at least 70% were shown as "X" at corresponding nodes.

7. TABLES

Table 1. Transcriptome profiles of *P. insidiosum* growing at body (37 °C or BT) and room (28 °C or RT) temperatures.

Table 2. The KEGG metabolic pathways existed in *P. insidiosum* (*S. cerevisiae* was the reference organism).

Table 3. Top 30 functional domains of *P. insidiosum* proteins by Interproscan analysis

Table 4. Thermally up- and down-regulated proteins of *P. insidiosum* (n = 1,074). The proteins can be divided into 309 groups, and the top 40 groups (based on number of proteins) were listed below (Q-value cut off < 0.05; the blanket indicates the predominate set).

Table 5. Top 40 groups of the MvirDB-matched virulence factors of *P. insidiosum* (VFID, MvirDB-defined virulence factor identification).

Table 6. Top 35 MvirDB-matched and thermally up-regulated proteins of *P. insidiosum* (Q-value cut off < 0.01; VFID, MvirDB-defined virulence factor identification).

Table 7. Putative RXLR effectors of *P. insidiosum*.

Table 8. The present of effectors (crinkler, RXLR, and elicitin) in *P. insidiosum*, other oomycetes, and diatoms.

Table S1. BLASTP search results of the predicted proteins of *P. insidiosum*. All protein sequences of the combined BT-RT dataset were searched for significant similarities (E-value cut-off $\leq 1e^{-4}$), utilizing the BLASTP algorithm and the NCBI's non-redundant protein database.

Table S2. A total of 1,074 genes of *P. insidiosum* growing at body temperature (BT), were significantly (Q-value < 0.05) up-regulated (625 genes) or down-regulated (449 genes), in comparison with the growth at room temperature (RT).

Table S3. All 24,610 predicted proteins of *P. insidiosum* were BLAST searched against the MvirDB databases and 1,974 *P. insidiosum* proteins matched 488 VFIDs (MvirDB-defined virulence factor identification).

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