รายงานวิจัยฉบับสมบูรณ์

โครงการ: Use of Effector-Encoding Genes to Explore Biology, Pathogenesis and Evolution of the Human-Pathogenic Oomycete *Pythium insidiosum*
(การใช้ effector genes เพื่อศึกษาชีววิทยา การก่อโรค และวิวัฒนาการของเชื้อ *Pythium insidiosum*)

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บทคัดย่อ

เชื้อ *P. insidiosum* เป็นเชื้อในกลุ่ม oomycetes ที่สามารถก่อโรคติดเชื้อ pythiosis ในคนและในสัตว์ การวินิจฉัยทำได้ยาก การรักษายังเป็นปัญหาเนื่องจากไม่มียาชนิดใดใช้รักษาได้ผล ถึงแม้ว่าเชื้อ *P. insidiosum* จะมีลักษณะเป็สายรา แต่เชื้อชนิดนี้กลับมีความสัมพันธ์ใกล้ชิดทางพันธุศาสตร์กับไดอะตอมและสาหร่ายมากกว่าเชื้อรา จากการศึกษาทางชีวพันธุศาสตร์ สามารถแบ่งเชื้อ *P. insidiosum* ออกเป็น 3 กลุ่มตามพื้นที่ๆแยกเชื้อได้ คือ Clade-I (ประเทศในทวีปอเมริกา), Clade-II (ประเทศในทวีปเอเชียและออสเตรเลีย), และ Clade-III (ประเทศไทย) ข้อมูลต่างๆเกี่ยวกับเชื้อ *P. insidiosum* ยังมีน้อยมาก ในบรรดาเชื้อก่อโรคชนิดอื่นจำนวนมาก จะปล่อยโปรตีนชนิด effectors ออกมา ซึ่งสามารถมีผลต่อระบบการตอบสนองของโฮสและส่งเสริมการติดเชื้อได้ โปรตีน elicitins เป็น effectors ชนิดหนึ่ง ที่พบเฉพาะในเชื้อกลุ่ม oomycetes ทั้งนี้ ในเชื้อ oomycetes ที่ก่อโรคในพืช พบว่า elicitins มีหน้าที่กระตุ้นระบบป้องกันตัวของพืช และตัวโปรตีนเองสามารถเกาะกับโมเลกุลกลุ่ม sterol ได้ เมื่อไม่นานมานี้ คณะผู้วิจัยสามารถค้นพบยีนจำนวนหนึ่งที่ถอดรหัสเป็น elicitins จากข้อมูลพันธุศาสตร์ของเชื้อ *P. insidiosum* โดยยังไม่ทราบหน้าที่ของยีนเหล่านี้ต่อการติดเชื้อในคน คณะผู้วิจัยจึงสนใจศึกษาและวิเคราะห์ยีน elicitin ทางด้านพันธุศาสตร์ ชีวเคมี และภูมิคุ้มกันวิทยา คณะผู้วิจัยสนใจยีน elicitin ยีนหนึ่งซึ่งมีการแสดงออกของยีนสูงที่อุณหภูมิร่างกาย และตั้งชื่อยีนนี้ว่า *ELI025* จากการศึกษา พบว่า *ELI025* ถอดรหัสเป็นโปรตีน ELI025 ซึ่งมีขนาดเล็ก มีองค์ประกอบเป็นไกลโคโปรตีน และถูกปล่อยออกมาจากเซลล์ของเชื้อ โดยโปรตีนนี้สามารถหลบหลีกการจับของแอนติบอดีได้ นอกจากนี้ โปรตีน ELI025 จากเชื้อ *P. insidiosum* สายพันธุ์ต่างๆ ที่แยกได้จากคน สัตว์ และสิ่งแวดล้อม มีความแตกต่างกันทางชีวเคมีและภูมิคุ้มกันวิทยา เนื่องจาก ELI025 เป็นโปรตีนที่พบเฉพาะใน *P. insidiosum* และไม่พบในเชื้อชนิดอื่นๆ ที่ก่อโรคในคน คณะผู้วิจัยจึงได้นำแอนติบอดีต่อโปรตีน ELI025 ที่สร้างได้จากกระต่าย มาใช้พัฒนาเป็นชุดตรวจชนิด immunohistochemical assay (IHC) เพื่อวินิจฉัยโรค pythiosis ในเนื้อเยื่อที่ติดเชื้อ ชุดตรวจ IHC สามารถตรวจพบเชื้อ *P. insidiosum* จากเนื้อเยื่อในกลุ่มทดสอบทั้งหมด 38 ตัวอย่าง (ความไวของชุดตรวจ 100%) โดยไม่ย้อมติดเชื้ออื่นๆ ในเนื้อเยื่อกลุ่มควบคุมจำนวน 49 ตัวอย่าง (ความจำเพาะของชุดตรวจ 100%) นอกจากนี้ คณะผู้วิจัยได้นำ effectors ชนิดต่างๆ ที่รวมกันอยู่ในโปรตีนที่เรียกว่า culture filtrate antigens ซึ่งสกัดได้จากเชื้อ *P. insidiosum* มาใช้พัฒนาชุดตรวจชนิด protein A/G-based immunochromatographic test (ICT) เพื่อตรวจหาแอนติบอดีต่อเชื้อในซีรัมผู้ป่วยและสัตว์ที่ป่วยเป็นโรค pythiosis ในการประเมินชุดตรวจ ICT ใช้ซีรัมจากกลุ่มทดสอบจำนวน 85 ตัวอย่าง (จากคน 28 ราย, สุนัข 24 ตัว, ม้า 12 ตัว, กระต่าย 12 ตัว, และโค 9 ตัว) และใช้ซีรัมจากกลุ่มควบคุมจำนวน 143 ตัวอย่าง (จากคน 80 ราย และสัตว์ 63 ตัว ที่ปกติหรือป่วยเป็นโรคอื่น) พบว่า ICT เป็นชุดตรวจที่อ่านผลได้เร็ว ใช้ง่าย และมีประสิทธิภาพ (ความจำเพาะ 100% และความไว 91%) ต่อการวินิจฉัยโรค pythiosis ในคนและสัตว์ โดยสรุป การศึกษาโปรตีน ELI025 อย่างละเอียดทำให้เข้าใจชีววิทยาและพยาธิกำเนิดของเชื้อ *P. insidiosum* มากขึ้น และนำมาซึ่งการใช้ประโยชน์จากโปรตีน ELI025 เพื่อพัฒนาชุดตรวจวินิจฉัยโรค pythiosis

คำสำคัญ: *Pythium insidiosum*, Pythiosis, Effector, Pathogenesis, Evolution, Diagnosis

Abstract

Pythium insidiosum is a unique oomycete that can infect humans and animals, and causes a life-threatening infectious disease, called “pythiosis”. Diagnosis of pythiosis is difficult. Controlling an infection caused by *P. insidiosum* is problematic because effective antimicrobial drugs are not available. Although *P. insidiosum* shares hyphal morphology with fungi, this pathogen is more closely related to diatoms and algae. Phylogenetic analyses divide *P. insidiosum* into 3 groups, according to geographic origins: Clade-I (Americas), Clade-II (Asia and Australia), and Clade-III (Thailand). Basic biological information of *P. insidiosum* is very limited. Many pathogens secrete proteins, known as effectors, which can affect the host response and promote the infection process. Elicitins are effector proteins found only in the oomycetes. In plant-pathogenic oomycetes, elicitins function as pathogen-associated molecular pattern molecules, sterol carriers, and plant defense stimulators. Recently, we reported a number of elicitin-encoding genes from the *P. insidiosum* transcriptome. Function of elicitins during human infections is unknown. One of the *P. insidiosum* elicitin-encoding genes, *ELI025*, is highly expressed and up-regulated at body temperature. This study aims to characterize the genetic, biochemical, and immunological properties of ELI025. We found that ELI025 is a small, abundant, secreted glycoprotein that can evade host antibody responses. We investigated geographic variation of ELI025 in 24 *P. insidiosum* strains isolated from humans, animals, and the environment. ELI025 was secreted by all *P. insidiosum* strains isolated from different hosts and geographic origins, but the protein had different biochemical and immunological characteristics. Since ELI025 has been identified in *P. insidiosum*, but not other human pathogens including true fungi, this study also aims to develop an immunohistochemical assay (IHC), using rabbit anti-ELI025 antibody (anti-ELI), for diagnosis of pythiosis. Thirty-eight *P. insidiosum* histological sections stained positive by the anti-ELI based IHC, to give 100% detection sensitivity. The IHC reported negative stains for all 49 negative control sections, to give 100% detection specificity. We also used the pool of effector proteins in culture filtrate antigens to develop a protein A/G-based immunochromatographic test (ICT), for detection of anti-*P. insidiosum* antibody in patient sera. Eighty-five serum samples from 28 patients, 24 dogs, 12 horses, 12 rabbits, and 9 cattle with pythiosis, and 143 serum samples from 80 human and 63 animal subjects in a healthy condition, with thalassemia, or with other fungal infections, were recruited for assay evaluation. Detection specificity and sensitivity of ICT were 100% and 91%, respectively. The ICT is a rapid, user-friendly, and efficient assay for serodiagnosis of pythiosis in humans and animals. In conclusion, comprehensive characterization of ELI025 promoted better understanding on biology and pathogenesis of *P. insidiosum*. ELI025 was an efficient target for development of the diagnostic assays (i.e., IHC and ICT) for pythiosis.

Keywords: *Pythium insidiosum*, Pythiosis, Effector, Pathogenesis, Evolution, Diagnosis

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1. Introduction

1.1 Background and significance of pytihosis:

Members in the genera *Pythium*, *Albugo*, *Peronospora*, *Hyaloperonospora, Phytophthora*, *Plasmopara*, *Bremia*, *Aphanomyces*, *Lagenidium* and *Saprolegnia* form a unique group of microorganisms, called "Oomycetes" (**Figure 1-1**), which belong to the Stramenopiles of the supergroup Chromalveolates (1,2). Although oomycetes share hyphal morphology with the fungi, molecular phylogenetic analysis shows that they are more closely related to diatoms and algae (3). Most pathogenic oomycetes infect plants, while a few species of the genera *Pythium*, *Aphanomyces*, *Lagenidium*, and *Saprolegnia* are capable of infecting animals (1). Controlling infections caused by the oomycetes is problematic because effective antimicrobial drugs are not available.

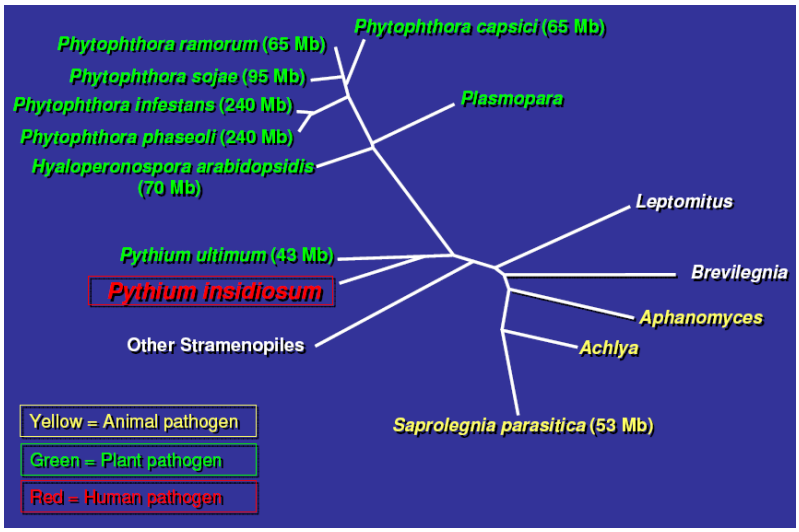

Figure 1-1. Phylogenetic relationship and genome size estimation of oomycete microorganisms (adapted from the figure kindly provided by Dr. Brett Tyler).

P. insidiosum is the unique oomycete that infects humans, and the resulting disease, called pythiosis, is devastating and often fatal (1,4). How *P. insidiosum* has evolved to become a successful human pathogen remains largely unknown. In nature, *P. insidiosum* inhabits swampy areas and presents in 2 forms (**Figure 1-2)**; mycelia and biflagellate zoospore which, after conversion to the hyphal form, initiates infection (5). The route of entry for *P. insidiosum* is direct contact to host surfaces (i.e., skin, eye). Pythiosis in animals has been reported in tropical and subtropical areas of the world (**Figure 1-3**) (4,6–12). Pythiosis in humans has been reported mostly from Thailand (**Figure 1-4**), and sporadic cases have been reported from U.S.A., Brazil, Haiti, New Zealand, Australia, Israel, India, and Malaysia (4,12,13). In Asian countries, where climate and geographic conditions are suitable for *P. insidiosum*, human cases of pythiosis are not expected to be rare. There are three phylogenetic groups associated with the geographic origins of the organism: clade-I containing only American isolates, clade-II containing American, Asian and Australian isolates, and clade-III containing American and Thai isolates (14).

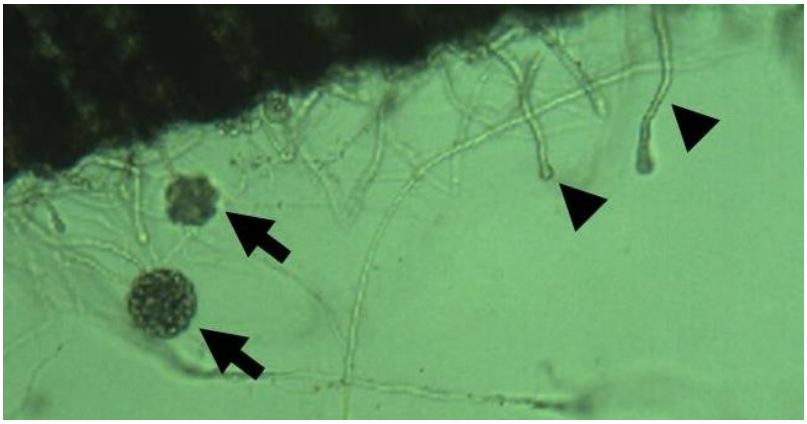

Figure 1-2. **Microbiological features of *P. insidiosum* colonized on a water plant:** Hyphae (arrow heads) and zoospore sac (arrows) containing up to 60 motile zoospores (picture courtesy of Dr. Piriyaporn Chongtrakool).

Many physicians and microbiologists are not familiar with pythiosis because it is a relatively new infectious disease. Pythiosis can be confused with other fungal infections (such as aspergillosis and zygomycosis) because of the similar microscopic characteristics of the causative pathogens and the similar clinical features (15). The two most common forms of pythiosis described in humans are vascular (**Figure 1-5A and 5B**) and ocular (**Figure 1-5C and 5D**) pythiosis. Arterial insufficiency syndrome and gangrenous ulceration of lower extremities are the main features of vascular pythiosis (12). Ocular pythiosis usually presents itself as a corneal ulcer. A striking comorbidity exists for vascular form of pythiosis: all patients have an underlying hematological disorder, 85% of which were cases of thalassemia. Iron overload is a major pathophysiological change resulting from the disease process and treatment of thalassemic patients and could be a factor that enhances host susceptibility to pythiosis. In contrast to vascular pythiosis, the majority of patients with ocular pythiosis (84% of cases) report no underlying medical condition, indicating that healthy individuals are susceptible to ocular infection (12,16).

Figure 1-3. Geographic distribution of pythiosis reported from around the world (4).

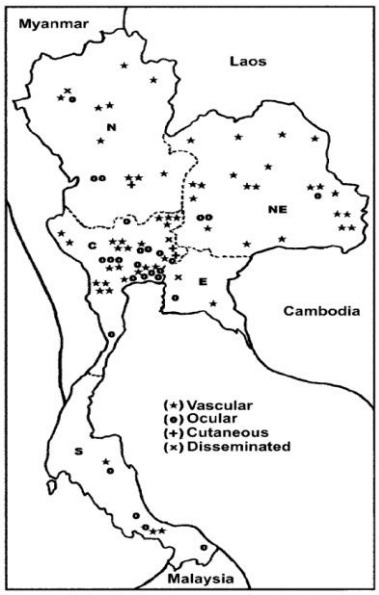

Figure 1-4. Geographic distribution of human pythiosis reported from Thailand (12).

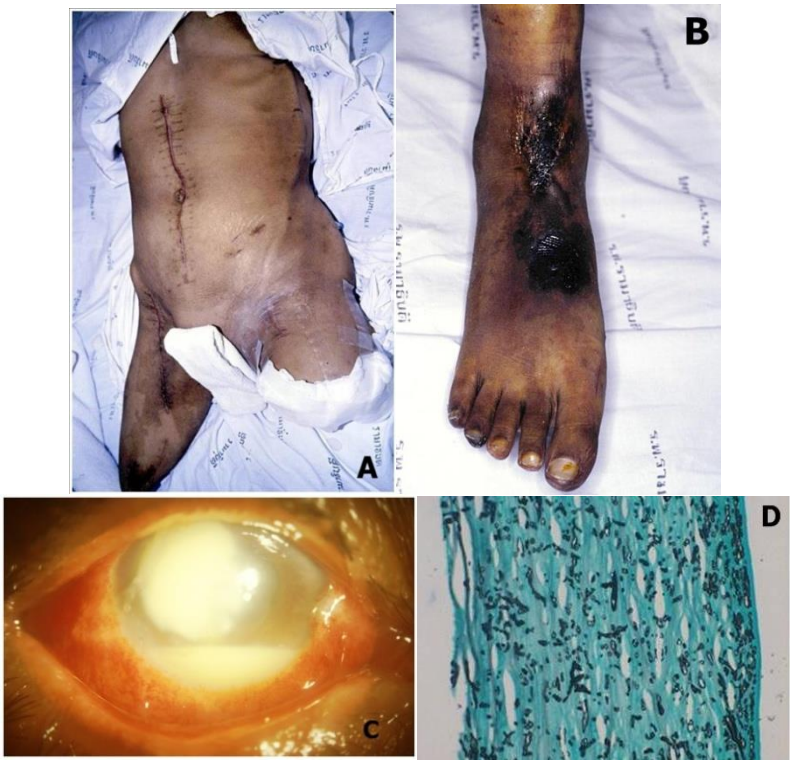

Figure 1-5. Clinical features of human pythiosis: (A) a patient with vascular pythiosis that has undergone bilateral leg amputation and infected aorta resection to control the infection; (the picture is kindly provided by Dr. Boonmee Sathapatayavongs); **(B)** gangrenous ulcers on foot of a pythiosis patient caused by advanced vascular infection (19); **(C)** corneal ulcer and marked inflammation of eye from patient with ocular pythiosis (16); **(D)** tissue section of corneal stroma full with *P. insidiosum* hyphal elements derived from a patient with ocular pythiosis (16).

Conventional antifungal agents are not effective for treatment of *P. insidiosum* infection, which likely due to the oomycetes lack the drug-targeted ergosterol biosynthesis pathway (12,17). The only treatment approach for pythiosis is radical surgery. As a result of advanced, uncontrollable and aggressive infection, at least 80% of vascular pythiosis patients undergo leg amputations and 40% die, while ~80% of ocular pythiosis patients undergo enucleation resulting in loss of eye sight. Immunotherapy using the crude extract prepared from *P. insidiosum* culture has limited efficacy for treatment of pythiosis (12,18).

In conclusion, human pythiosis is endemic in Thailand, it has high rate of morbidity and mortality, thalassemia is a predisposing factor, treatment of pythiosis is difficult, and basic information of pythiosis and its causative agent is lacking. Thus, it is apparent that more needs to be done in the way of basic research to provide insights into biology, pathogenesis, and evolution of *P. insidiosum*, and thereby lead to the discovery of novel strategies for infection control, such as, new antimicrobial agents and vaccines.

1.2 *Pythium insidiosum* effectors:

Because genetic information of *P. insidiosum* is limited, we used the 454 sequencing platform to generate *P. insidiosum* transcriptome, comprising 26,735 genes, which can be theoretically translated to 24,610 proteins. Potential virulence factors of *P. insidiosum* were identified from this transcriptome. We are interested in a group of putative virulence factors that forms “effectors”. The effectors are secreted molecules that manipulate host cell structure and function, in order to facilitate infection process of many pathogens, including oomycetes (20). Based on target sites, effectors can be simply divided into two groups: extracellular effectors which are secreted and interacted with extracellular targets, and intracellular effectors which are secreted and entered host cells (20). We have found *P. insidiosum*’s genes that significantly matched the extracellular effectors, called elicitins (**Table 1-1**).

Table 1-1. Elicitin effectors of *P. insidiosum*, other oomycetes and diatoms.

Microorganism	Genome size (Mb)	Elicitins	References
Pythium insidiosum	53	61	(21)
Pythium ultimum	43	24	(22)
Phytophthora infestans	240	40	(22,23)
Phytophthora sojae	95	57	(23,24)
Phytophthora ramorum	65	50	(23,24)
Hyaloperonospora arabidopsidis	100	15	(25)
Albugo candida	45	9	(26)
Aphanomyces euteiches	N/A	1	(27)
Phaeodactylum tricornutum (diatom)	27	-	(22,28)
Thalassiosira pseudonana (diatom)	35	-	(22,29)

Elicitins are a large protein family with the conserved elicitin domain (~80-100 amino acids long with six cysteine residues), initially reported in *Phytophthora* and *Pythium* species (30–32). A striking observation about the *P. insidiosum* effectors was the discovery of a remarkably-extensive repertoire of elicitins in the transcriptome. Less than 60 eliticin proteins can be found in other oomycetes, for example, 57 in *Phytophthora sojae*, 24 in *Pythium ultimum*, and only one in *Aphanomyces euteiches* (**Table 1-1**). *P. insidiosum* harbored ~300 elicitins, which were at least 5 folds greater than that found in other oomycetes (**Table 1-1**). Alignment of the 98 full-length elicitin domain sequences of *P. insidiosum* showed high degree of similarity (**Figure 1-6A**). Five different protein organizations were observed in the *P. insidiosum* elicitins (**Figure 1-6B**). Elicitins can trigger defense responses and programmed cell death of host plant cells, in order to promote infection by some pathogenic oomycetes (30). They are recognized as a pathogen-associated molecular pattern (PAMP) by plants (33). The elicitins can act as sterol-carrying proteins, in order to acquire exogenous sterols for growth of some oomycetes (i.e., *Phytophthora* and *Pythium* species) which lack endogenous ergosterol biosynthesis pathway (34–36). Based on homology modeling, we predicted that *P. insidiosum*'s elicitins can bind cholesterol and ergosterol (**Figure 1-7**) (37). Elicitin domain sequences from *P. insidiosum* (n=98) and other oomycetes (n=38) can be phylogenetically divided into 10 distinct clades (**Figure 1-8**). Clade-A formed the biggest group of elicitins which all belong to *P. insidiosum*. It should be noted that elicitins are not present in other groups of organisms, but oomycetes (30). Therefore, among human pathogens, the elicitin can be considered as a molecular signature of *P. insidiosum*. Many *P. insidiosum*'s elicitins were predicted to be secreted (present of signal peptide) or cell-wall associated (present of GPI anchor or glycosylation site) proteins (30), making them a good candidate for development of a more effective diagnostic or therapeutic tools.

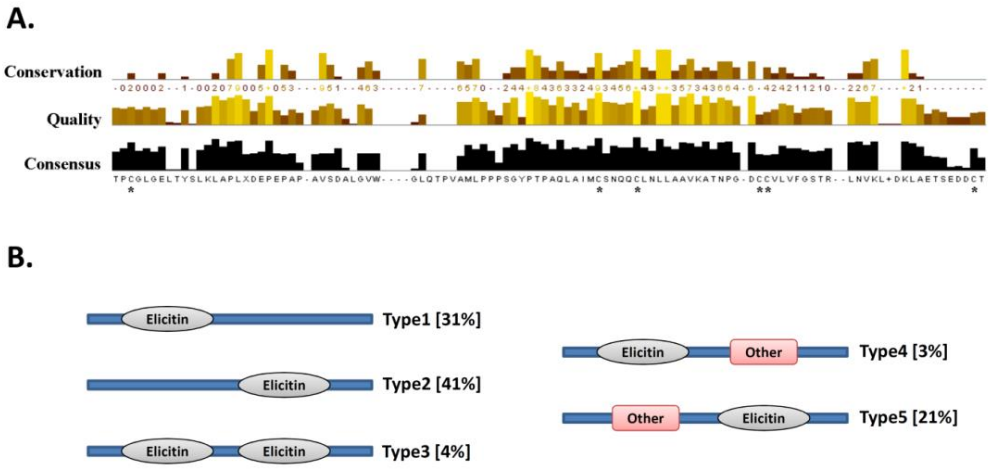

Figure 1-6. Sequence alignment and domain organization of the elicitin proteins: **(A)** Alignment of the 98 unique elicitin domain sequences, using the Clustal Omega, showed a high degree of sequence conservation (upper bar graph). Alignment quality of each amino acid residue (depends on number and type 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).

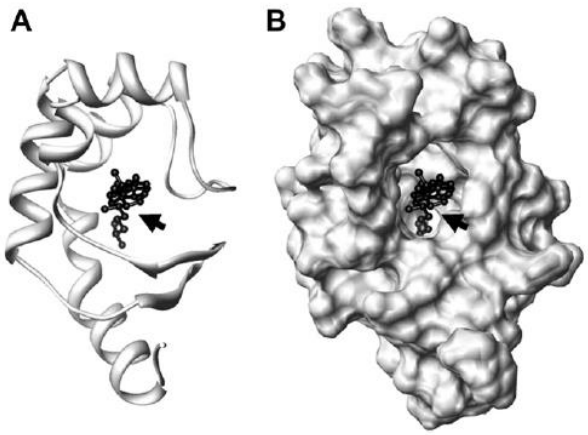

Figure 1-7. Homology modeling of a putative elicitin of *P. insidiosum* in complex with one molecule of cholesterol (shown as ball and stick model, indicated by an arrow), which is encapsulated in the pocket-like cavity (37): **(A)** Ribbon structure model, and **(B)** Surface structure model.

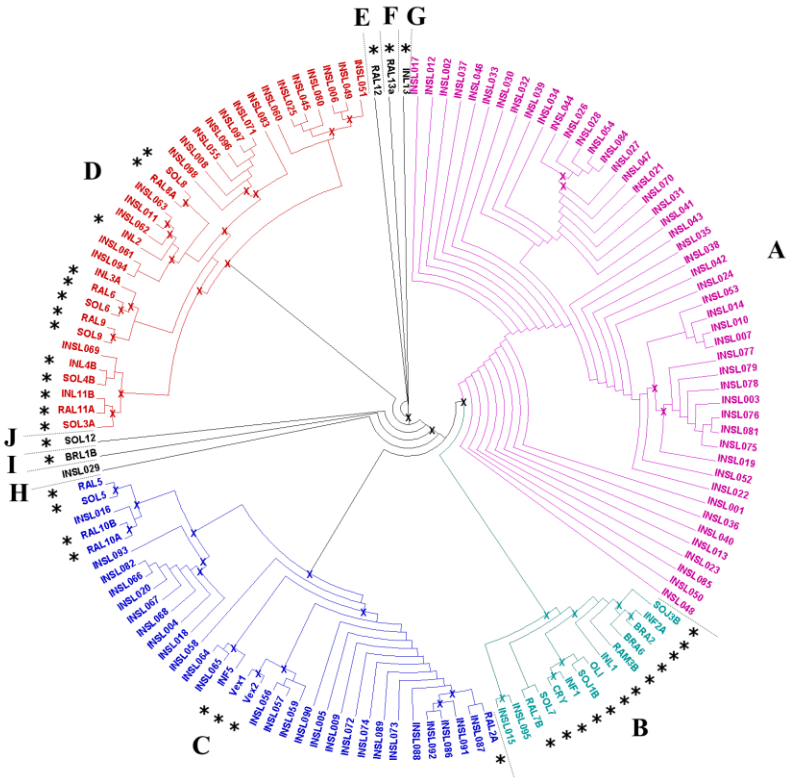

Figure 1-8. Phylogenetic analysis of oomycete elicitins (unpublished data): The maximum likelihood method was used to generate a phylogenetic tree of the 134 unique full-length elicitin domain sequences from *P. insidiosum* (n=98; INSL001-098) and other oomycetes (n=38; indicated by asterisks). The tree shows 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% are shown as “X” at corresponding nodes.

Gene gain, gene loss and gene modification impact the presence or absence of effectors in oomycetes. Such evolutionary processes should be required for specialization of lifestyle, host specificity, and virulence strategy of each pathogenic oomycete. We have now generated the genome and transcriptome databases which are invaluable resources for exploring biology, pathogenesis, and evolution of *P. insidiosum*. Comprehensive sets of *P. insidiosum*'s putative effectors were identified. Further studies of the effector molecules could provide an insight into pathogenesis mechanism of *P. insidiosum*. In a medical stand point, the putative effectors of *P. insidiosum* could prove to be useful for development of better diagnostic and therapeutic tools for pythiosis.

1.3 Objectives of the study:

- To characterize effector-encoding genes of *P. insidiosum*
- To assess role of *P. insidiosum* effectors in host immune responses
- To explore evolution of *P. insidosum* by using effector-encoding genes
- To develop *P. insidiosum* effector-based diagnostic assays

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2. Project details

2.1 Characterization of the effector protein, ELI025, of *Pythium insidiosum*

2.1.1 Introduction

Many pathogenic microorganisms secrete proteins that promote infection by interfering with host cell function and altering host responses [14-22]. For example, the bacterium *Helicobacter pyroli* secretes CagA to perturb a host cell signaling pathway, and leads to development of peptic ulcer [17,18]. The malarial parasite *Plasmodium falciparum* secretes some histidine-rich proteins that facilitate its survival inside red blood cells [19]. In many plant-pathogenic oomycetes, the multifunctional elicitin molecules facilitate infection by triggering host tissue necrosis [22]. The elicitin can also be recognized as a pathogen-associated molecular pattern by plant cells [23-26], and serve as a sterol-carrying protein for acquiring exogenous sterols [27-33].

Recent transcriptome analyses revealed that *P. insidiosum* harbors an extensive repertoire of elicitin-domain-containing proteins (~300 proteins), many of which (~60 proteins) are predicted to be secreted [34,35]. The biological role of elicitin in human hosts is unknown. The *P. insidiosum* elicitin-encoding gene, *ELI025*, is highly expressed and 5-fold up-regulated when *P. insidiosum* hyphae is grown at body temperature (37 °C), compared to hyphae grown at room temperature (28 °C) [34,35], suggesting that *ELI025* may be required for survival of *P. insidiosum* inside a human host. The current study reports on the cloning and expression of *ELI025* for genetic, biochemical and immunological analyses. Molecular characterization of elicitin is a significant step in exploring the biology and virulence of this understudied microorganism and could lead to new strategies for infection control.

2.1.2 Materials and Methods

Microorganisms: The *P. insidiosum* strains Pi-S, MCC18, and P01, were obtained from a collection of microorganisms that were isolated from clinical samples received for routinely culture identification. All strains were maintained on Sabouraud dextrose agar at room temperature and sub-cultured once a month.

Serum samples: Three serum samples were obtained from pythiosis patients diagnosed by culture identification or serological tests [36-41]. To serve as controls, three serum samples were obtained from healthy blood donors who came to the Blood Bank Division, Department of Pathology, Ramathibodi Hospital. Rabbit anti-rELI025 sera were purchased from the Biomedical Technology Research Laboratory, Faculty of Associated Medicine, Chiang Mai University, Thailand. To block the rabbit anti-rELI025 antibodies from the rabbit serum, 20 µl of rELI025 (2.4 mg/ml) and 1.5 ml of diluted rabbit serum [1:2,000 in 5% skim milk in TBS (pH 7.5)] were co-incubated with gentle agitation at 4°C overnight. All sera were kept at -20 °C until use.

Protein preparation: Crude protein extracts of *P. insidiosum*, including soluble antigen from broken hyphae (SABH; containing intracellular proteins) and culture filtrate antigen (CFA; containing secreted proteins), were prepared according to the methods described by Chareonsirisuthigul et al [41]. Briefly, 100 ml Sabouraud dextrose broth was inoculated from an actively growing *P. insidiosum* colony and incubated, with shaking (~150 rpm), at 37 ºC for 10 days. The organism was killed with 0.02% Thimerosol (Sigma). Hyphae were collected by filtration on a 0.22-µm-pore-size membrane (Durapore, Merck Millipore), and ground in a mortar with pre-cooled distilled water (1.5 g hyphae per 30 ml water). Supernatant, following centrifugation (10,000 x g) of the cell lysate at 4 ºC for 30 min, was filtered through a 0.22-µm-pore-size membrane (Durapore, Merck Millipore).

Both filtered supernatant (SABH) and cell-free broth (CFA) were 100-fold concentrated by ultrafiltration (10,000 molecular weight cut-off membrane; Amicon Ultra 15M, Merck Millipore). Protein concentration was measured by Bradford's assay [42]. SABH and CFA were stored at -20 °C until use.

Genomic DNA extraction: *P. insidiosum* genomic DNA (gDNA) was extracted using the modified method of Lohnoo et al [43]. Briefly, hyphal mat (~500 mg) was transferred to a 2-ml tube containing glass beads (~1,000-μm diameter; Sigma) and 400 μl of homogenizing buffer [0.4 M NaCl, 10 mM Tris–HCl (pH 8.0), 2 mM EDTA (pH 8.0)]. The tube was shaken at 30 Hz for 2 min, in a tissue homogenizer (TissueLyzer, QIAGEN), before adding 20% sodium dodecyl sulfate (final concentration, 2%) and proteinase K (final concentration, 400 μg/ml). The cell lysate was incubated, with gentle inversion, at 55 ºC, overnight. The sample was then mixed with 300 μl of 6 M NaCl, vigorously vortexed for 30 s, and centrifuged (10,000 x g) at room temperature for 30 min. The supernatant was then mixed with an equal volume of isopropanol, incubated at -20ºC for 1 hr, and centrifuged (12,000 x g) at 4 ºC for 20 min. The gDNA pellet was collected and washed with 70% ethanol, air dried, and resuspended in sterile water. All extracted gDNAs were kept at -20 °C until use.

Plasmid construction: The full-length ELI025-encoding sequence (NCBI accession number: HS975204) was amplified from the pCR4-blunt-TOPO vector harboring PinsEST#025 cDNA [34], in a 50-μl PCR reaction containing 1.5 μl of PCR product, 1 μl of the Elongase and its buffer mixture (buffer A:B ratio = 1:4) (Invitrogen), 200 μM of dNTPs, and 0.4 μM each of the primer ELI025_NdeI (5'-GGCATCACATATGTACAACGAGACCAAGCCG-3') and ELI025_EcoRI (5'-CAAGAATTCCTAGGCCTTGCAGCTCGTC-3'). The reaction was carried out in a MyCycler (Biorad) with the following conditions: initial denaturation at 94°C for 30 s, 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 68°C for 1.10 min, and final extension at 68°C for 5 min. The PCR product was double digested with *NdeI* and *EcoRI* (New England Biolabs), and directionally cloned into pET28b (Novagen), yielding an in-frame His-tag fusion on the N-terminus of ELI025. The resulting plasmid, pET28b-ELI025 (**Figure 2.1-1A**), was propagated in the *Escherichia coli* strain DH5α. The sequence of the ELI025-coding region of the plasmid was confirmed using primers, T7-promoter (5'-TAATACGACTCACTATAGGG-3') and T7-terminator (5'-GCTAGTTATTGCTCAGCGG-3').

Protein expression and purification: The recombinant ELI025 protein (rELI025; plasmid pET28b-ELI025) was expressed from the *E. coli* strain rosetta-gami2 (DE3) (Novagen). A clone harboring pET28b-ELI025 was grown in the Terrific broth [44], supplemented with tetracycline (12.5 μg/ml), chloramphenicol (34 μg/ml), and kanamycin (30 μg/ml), until the cells reached 0.5 optical density. IPTG (final concentration, 1 mM; Omnipur) was added, before further shaking incubation (250 rpm) at 25 °C for 12 hr. The culture was centrifuged (6000 x g) at 4 °C for 10 min and the pellet was resuspended in binding buffer [20 mM sodium phosphate buffer (pH 7.4) and 0.1 M NaCl] (1 g pellet per 5 ml binding buffer), mixed with lysozyme (final concentration, 1 mg/ml; BioBasics), incubated on ice for 30 min, sonicated (setting: 50% amplitude, 20 cycles, 10/10-second pulse on/off), and centrifuged (10,000 x g) at 4°C for 30 min. The resulting supernatant was applied to a HiTrap IMAC FF column (GE healthcare), pre-charged with 0.1 M $NiCl_2$. The column was sequentially washed with the binding buffer containing 60 and 100 mM imidazole. Protein was eluted from the column with binding buffer containing 500 mM imidazole. The concentration of the purified recombinant protein was determined by Bradford's assay [42], and kept at -30 °C until use.

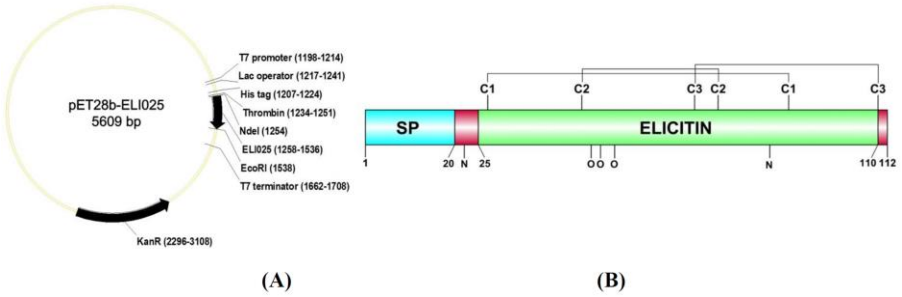

Figure 2.1-1. Cloning and expression of *ELI025*: (**A**) Plasmid DNA map of pET28b-ELI025 shows the cloning sites (*Nde*-I and *EcoR*-I) of *ELI025*. Expression of *ELI025* is under the control of the T7 promoter. The numbers in parentheses indicate a location of each plasmid component; (**B**) Protein structure of ELI025 shows a signal peptide (SP; amino acid position 1-20), an elicitin domain (amino acid position 25-110), three disulfide bonds (C1, cysteine position 27 and 91; C2, cysteine position 47 and 76; C3, cysteine position 71 and 110), two predicted N-linked glycosylation sties (N; amino acid position, 22 and 87), and three predicted O-linked glycosylation sties (O; amino acid position 49, 51, and 54).

SDS-PAGE and Western blot: SABH, CFA, and rELI025 were separated by SDS-PAGE (4% stacking and 12% separating gel) at 150 V, for 65 min, using the Mini-PROTEAN II apparatus (Biorad). Proteins were stained with either Coomassie blue R-250 or Silver staining kit (Thermo Scientific). The Image Lab 3.0 program (Biorad) was used to estimate protein molecular weight (kilo Dalton; kDa) based on migration of pre-stained broad range protein markers (Biorad). For Western blot analysis, the separated proteins were transferred and immobilized onto a 0.2-µm-pore-size PVDF membrane (Merck Millipore), using the Biorad Mini Trans-Blot cell (setting: 100 V for 60 min). The blotted membrane was blocked with 5% skim milk (Sigma) in blocking buffer [TBS; 150 mM NaCl, 10 mM Tris-HCl (pH 7.5)] at 4 °C, overnight, or room temperature for an hour. The membrane was washed 3 times with the washing buffer [TTBS; 500 mM NaCl, 20 mM Tris-Cl, and 0.1% Tween-20 (pH 7.5)]. The membranes were incubated with the primary antibodies diluted in the blocking buffer [1:1,000 for mouse anti-6x histidine antibody (Merck Millipore); 1:2,000 for rabbit anti-rELI025 serum; 1:1,000 for patient serum] for 2 hr at room temperature (anti-6x histidine and anti-rELI025) or overnight at 4 °C (patient serum), and washed 3 times with TTBS. The secondary antibodies, diluted in the blocking buffer [1:2,000 for goat anti-mouse IgG conjugated with horseradish peroxidase (Merck Millipore); 1:5000 for goat anti-rabbit IgG conjugated with alkaline phosphatase (Southern Biotech); 1:3000 for goat anti-human IgG conjugated with horseradish peroxidase (Biorad)], were added to the membrane and incubated for 2-3 hr at room temperature. After washing the membrane 3 times with TTBS, substrate and chromogen (4CN and H_2O_2 for horseradish peroxidase; NBT and BCIP for alkaline phosphatase) were added to develop Western blot signals. Intensities of Western blot bands were quantitated by the GelQuant.NET software (http://biochemlabsolutions.com/GelQuantNET.html).

Mass spectrometric analysis: The 10- and 15-kDa bands present on SDS-PAGE gel and Western blot were excised from gel and PVDF membrane, respectively. Proteins were extracted and trypsin digested, using the method described by Shevchenko et al. [45]. The digested proteins were analyzed by an Ultimate 3000 LC System (Dionex, USA) coupled to an HCTultra PTM Discovery System (Bruker Daltonics Ltd., U.K.) at the Proteomics Research Laboratory, Genome Institute, National Center for Genetic Engineering and

Biotechnology, Thailand. The Bruker Daltonics Data Analysis version 4.0 (Bruker Daltonics Ltd., U.K.) was used to analyze raw mass spectrometric data. The MASCOT software (Matrix Science, UK) was used to search the obtained MS and MS/MS data against ~15,000 genome-derived predicted proteins of *P. insidiosum* (unpublished data).

Deglycosylation of glycoprotein: A glycoprotein deglycosylation kit (Calbiochem) was used to remove sugar moieties (N- and O-linked glycosylation) from the native ELI025 in CFA. Briefly, 50 μg of CFA, 10 μl of 5x kit deglycosylation buffer, 2.5 μl of the kit denaturation solution, and distilled water were mixed to the final volume of 50 μl. The mixture was heated at 100°C for 5 min and cooled down to room temperature, before adding 2.5 μl of 15% TRITON X-100. To remove N-glycosyl groups, 1 μl of N-glycosidase F was added to the reaction. To remove O-glycosyl groups, 1 μl of the enzyme mixture, including α-2-3,6,8,9-neuraminidase, endo-α-N-acetylgalactosaminidase, β1,4-galactosidase, and β-N-acetylglucosaminidase, was added to the reaction. The protein-enzyme mixture was incubated at 37 °C for 3 hr.

Immunohistochemical staining assay: Immunohistochemical staining assay was performed, using the method described by Keeratijarut *et al* [46], with some modifications. A paraffin-embedded tissue section (4-μm thickness) from a patient with vascular pythiosis was pretreated with xylene and absolute ethanol (Merck), before washing with phosphate buffered saline (PBS; pH 7.4). The tissue section was incubated with Tris-EDTA buffer (TE buffer; pH 9.0) at 95^{O}C for 40 min, treated with 10% H_2O_2 in PBS for 10 min, and washed with PBS. Then, the tissue section was incubated with 200 μl of either rabbit pre-immune serum or rabbit anti-rELI025 serum (1:16,000 in PBS) at 4^{O}C overnight, washed with PBS (5 min each), and incubated with 200 μl of mouse anti-rabbit IgG antibody conjugated with horseradish-peroxidase (Thermo Scientific, USA) for 30 min. To develop color, the substrate 3,3'-diaminobenzidine tetrahydrochloride (DAKO, USA) was added to the tissue section and incubated at room temperature for 5 min. The tissue section was counterstained with hematoxylin before examination with a light microscope (ECLIPSE Ci, Nikon, Japan).

Polymerase chain reaction and DNA sequencing: A draft genome sequence of the *P. insidiosum* strain Pi-S (unpublished data) was used to design primers for PCR amplification of the rELI025-encoding sequence and its promoter region (ELI025_promoter_F2, 5'-CATGGACAGCGTCATCTCTGG-3'; ELI025_promoter_R1, 5'-GCGTCAAGATGAGAAACGAGG-3'). Each amplification reaction was performed in a 50-μl reaction containing 100 ng of genomic DNA template, 0.02 U/μl of DNA polymerase (Phusion), 1x Phusion buffer, 200 μM of dNTPs, and 0.5 μM each of the primer. The amplification was carried out in a Mastercycler Nexus thermal cycler (Eppendorf), with the following conditions: an initial denaturation at 98 °C for 30 s, 35 cycles of denaturation at 98 °C for 10 s, annealing at 55 °C for 30 s, and extension at 72°C for 1 min, and a final extension at 72°C for 10 min. The PCR products were purified using a NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel) and assessed for amount and size by 1% gel electrophoresis.

Direct sequencing of PCR products was performed using a BigDye terminator V3.1 cycle sequencing kit (Applied Biosystems) and an ABI Prism 3100 Genetic Analyzer (Applied Biosystems). The primers used for sequencing included ELI025_F1 (5'-TACAACGAGACCAAGCCGTG-3'), ELI025_R1 (5'-GGCCTTGCAGCTCGTCTC-3'), ELI025_promoter_F2, ELI025_promoter_R1, ELI025_promoter_seqF1 (5'-CGCCCCCTTTCTTCCCGAC-3') and ELI025_promoter_seqR1 (5'-CCAACCAGACGCCGTCTG-3'). The sequences were analyzed using the ABI Prism DNA Sequencing Analysis software (Applied Biosystems, USA).

Bioinformatic analysis: The molecular weight of ELI025 was calculated by ProtParam [47]. Signal peptide, transmembrane domain, N- and O-linked glycosylation and GPI-anchor of ELI025 were predicted using the SignalIP program version 4.0 [48], the TMHMM program version [49], the NetNGlyc (http://www.cbs.dtu.dk/services/NetNGlyc), and NetOGlyc [50] programs, and the big-PI predictor [51], respectively. The promoter and ELI025-coding sequences from all *P. insidiosum* strains used in this study were aligned and compared using the ClustalX program version 2 and the GeneDoc program [52,53].

Homologous protein search: The elicitin domain sequence of ELI025 was used to BLAST search for elicitin homologous proteins encoded in the genomes and transcriptomes, or present in the proteomes of 18 oomycetes, 10 fungi, 4 algae, 3 diatoms, and one protozoan [54-72] (Table 2.1-1). The cut-off E-value for BLAST searches was $\leq 1 \times 10^{-4}$. If a BLAST search of particular genome database was not possible online, then a local BLASTP and TBLASTN search was performed using the BLAST 2.2.28+ program (http://www.ncbi.nlm.nih.gov/news/04-05-2013-blast-2-2-28/).

Phylogenetic analysis: Elicitin domain sequences from different oomycete organisms (**Table 2.1-1**) were analyzed online at http://www.phylogeny.fr/ [73]. The sequences were aligned by MUSCLE [74], and phylogenetic relationships were calculated by Neighbor-joining with 1,000 bootstraps [75] and the Jones-Taylor-Thornton matrix substitution model [76]. A phylogenetic tree was generated by TreeDyn [77].

Nucleotide sequence accession numbers: All ELI025-coding sequences from *P. insidiosum* strain Pi-S, MCC18, and P01 have been submitted to the DNA data bank of Japan (DDBJ), under accession numbers AB971191 to AB971193, respectively.

2.1.3 Results

Structures of *ELI025* and its gene product: The DNA sequence covering the 5'-untranslated region, coding sequence, and 3'-untranslated region of the *ELI025* gene was successfully PCR-amplified from gDNA of three different *P. insidiosum* strains: Pi-S (1,106-bp long; accession number, AB971191), MCC18 (1,056-bp long; accession number, AB971192), and P01 (1,036-bp long; accession number, AB971193). No intron was identified when the gDNA-derived (accession number, AB971191-3) and mRNA-derived (accession number, HS975204 and FX528334) ELI025-coding sequences were aligned. Analyses of the coding sequences for the *ELI025* alleles of three different strains of *P. insidiosum* by ClustalX version 2 [52] and GeneDoc [53] programs showed 98-99% identity and 99-100% similarity with each other (data not shown).

The 5'-untranslated and -flanking DNA sequences of the *ELI025* gene from the three *P. insidiosum* strains were compared with that of various genes from several oomycetes and parasites (**Figure 2.1-2)**. These sequences share a 19-nucleotide oomycete core-promoter sequence, located between 9 and 79 nucleotides upstream of the start codon (**Figure 2.1-2**). Two putative core-promoter components, an initiator element (Inr; 5'-TCATTCC-3') and a flanking promoter region (FPR; 5'-CAACCTTCC-3'), were identified in this region of *ELI025* (**Figure 2.1-2**). A predicted transcription start site (+1; **Figure 2.1-2**) of the *ELI025* gene is within the Inr element. A TATA box was not observed in the promoter region of *ELI025*.

Table 2.1-1. BLAST search of the ELI025 amino acid sequence against the genomes, transcriptomes, or proteomes of 18 oomycetes, 10 fungi, 4 algae, 3 diatoms, and 1 protozoan (the cut-off E-value $\leq 1 \times 10^{-4}$).

Organisms	Group	Subgroup	Number of BLAST hits	E-value of the best BLAST hit	References
Pythium ultimum	Oomycete	Pythiales	27	3.30E-31	[54]
Pythium aphanidermatum	Oomycete	Pythiales	20	1.00E-15	[54]
Pythium irregulare	Oomycete	Pythiales	18	2.00E-34	[54]
Pythium arrhenomanes	Oomycete	Pythiales	17	8.80E-16	[54]
Pythium iwayamai	Oomycete	Pythiales	14	7.00E-30	[54]
Pythium vexans	Oomycete	Pythiales	14	1.00E-20	[54]
Phytophthora sojae	Oomycete	Peronosporales	26	1.46E-22	[55]
Phytophthora ramorum	Oomycete	Peronosporales	25	1.00E-21	[55]
Phytophthora parasitica	Oomycete	Peronosporales	16	1.11E-14	BI [a]
Phytophthora capsici	Oomycete	Peronosporales	15	7.02E-20	[56]
Phytophthora cinnamomi	Oomycete	Peronosporales	14	5.51E-20	JGI [b]
Phytophthora infestans	Oomycete	Peronosporales	10	2.11E-16	BI [a]
Pseudoperonospora cubensis	Oomycete	Peronosporales	6	2.00E-07	[57]
Hyaloperonospora arabidopsis	Oomycete	Peronosporales	2	1.00E-06	[58]
Albugo laibachii	Oomycete	Albuginales	1	6.40E-13	[59]
Aphanomyces euteiches	Oomycete	Saprolegniales	-	-	[60]
Saprolegnia diclina	Oomycete	Saprolegniales	-	-	BI [a]
Saprolegnia parasitica	Oomycete	Saprolegniales	-	-	[61]
Phaeodactylum tricornutum	Diatom	Bacillariophyta	-	-	[62]
Pseudo-nitzschia multiseries	Diatom	Bacillariophyta	-	-	JGI [b]
Thalassiosira pseudonana	Diatom	Bacillariophyta	-	-	[63]
Aurantiochytrium limacinum	Microalgae	Labyrinthulida	-	-	JGI [b]
Nannochloropsis gaditana	Microalgae	Eustigmatophyceae	-	-	[64]
Aureococcus anophagefferens	Brown tide algae	Pelagophyceae	-	-	[65]
Ectocarpus siliculosus	Brown algae	PX clade	-	-	[66]
Blastocystis hominis	Protozoan	Blastocystis	-	-	[67]
Aspergillus spp.	Fungi	Ascomycota	-	-	[68]
Candida spp.	Fungi	Ascomycota	-	-	[69]
Fusarium oxysporum	Fungi	Ascomycota	-	-	BI [a]
Histoplasma capsulatum	Fungi	Ascomycota	-	-	BI [a]
Paracoccidioides brasiliensis	Fungi	Ascomycota	-	-	BI [a]
Pneumocystis jirovecii	Fungi	Ascomycota	-	-	[70]
Mucor circinelloides	Fungi	Zygomycota	-	-	BI [a]
Rhizopus delemar	Fungi	Zygomycota	-	-	BI [a]
Rhizopus oryzae	Fungi	Zygomycota	-	-	[71]
Cryptococcus neoformans	Fungi	Basidiomycota	-	-	[72]

Footnote:

[a] Broad institute genome database

[b] Genome portal of the Department of Energy Joint Genome Institute

The predicted full-length ELI025 protein sequences (112 amino acids long) from the three *P. insidiosum* strains were 100% identical. A predicted signal peptide of ELI025 covered the first 20 N-terminal amino acids **(Figure 2.1-1B)**. The calculated molecular weights of ELI025, with and without the signal peptide, were 12 and 10 kDa, respectively. The elicitin domain spanned from amino acid position 25 to 110. By analogy to known elicitins [34], three disulfide bonds (Cys27/Cys91, Cys47/Cys76, and Cys71/Cys110) were identified

within the elicitin domain of ELI025 (C1, C2, C3; **Figure 2.1-1B**). There are two predicted N-linked glycosylation sites at amino acid positions 22 and 87, and three predicted O-linked glycosylation sites at amino acid positions 49, 51, and 54 (**Figure 2.1-1B**). Neither a GPI anchor nor a transmembrane region are predicted for ELI025.

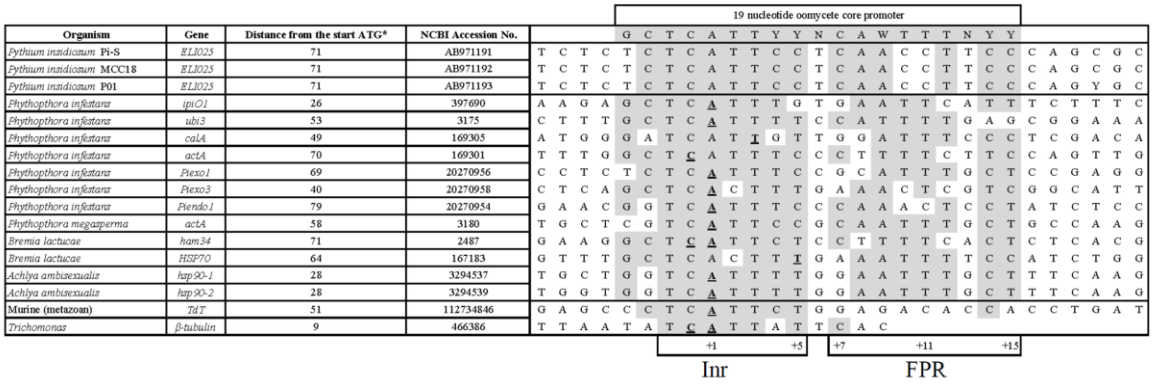

Figure 2.1-2. Sequence alignment of core promoter regions of the *P. insidiosum ELI025* gene and various genes from several oomycetes and parasites. The *ELI025* sequences (accession number AB971191-3), used for the alignment, are derived from three different *P. insidiosum* strains. Conserved nucleotides are highlighted in grey. The underlined letters indicate the known transcriptional start site, and is indicated below as "+1". Two putative core promoter components, an initiator element (Inr; 5'-TCATTCC-3'; positions -2 to +5) and a flanking promoter region (FPR; 5'-CAACCTTCC-3'; positions +7 to +15), are found in the upstream region of all genes. (Abbreviation: NCBI, National Center for Biotechnology Information).

Homologous proteins of ELI025: The ELI025 amino acid sequence was used for BLAST analyses of the genomes, transcriptomes, and proteomes of 36 different microorganisms (**Table 2.1-1**). No significant BLAST hit was identified in non-oomycete organisms. Three oomycetes (*A. euteiches*, *S. diclina*, and *S. parasitica*), which belong to the subgroup Saprolegniales, lacked sequences homologous to ELI025. A number of BLAST hits were found in 15 species of oomycetes, including *Pythium* spp. (14-27 hits), *Phytophthora* spp. (10-26 hits), *P. cubensis* (6 hits), *H. arabidopsis* (2 hits), and *A. laibachii* (1 hit). A signal peptide and an elicitin domain were identified in these top BLAST hit proteins (**Figure 2.1-3A**). The similarity between the ELI025 signal peptide and signal peptides of the other oomycetes' elicitins (17-23 amino acids long) was high (mean, 44%; median, 47%; range, 19-71%). Elicitin domain sequences of ELI025 and the other top BLAST hit proteins (83-94 amino acids long) contained 6 conserved cysteine residues (**Figure 2.1-3A**). Phylogenetic analysis, based on the elicitin domain sequences, divided the oomycetes into 5 closely related groups (Gr1-5): Gr1 contained all *Phytophthora* species; Gr2 and Gr3 comprised mainly *Pythium* species; Gr4 included *A. laibachii* and Gr5 had only *P. cubensis* (**Figure 2.1-3B**).

ELI025 is a major secreted non-immunogenic glycoprotein: The recombinant protein, rELI025, was successfully expressed and purified from *E. coli* (protein yield: 2 mg per 1 liter of bacterial culture). Purity of rELI025 was at least 99%, as demonstrated by silver staining analysis of SDS-PAGE gel. The molecular weight of rELI025 in the SDS-PAGE gel was estimated to be 12.4 kDa (**Figure 2.1-4A**). rELI025 appeared as an intense 12.4-kDa Western blot band, when reacted with the mouse anti-6x histidine-tag antibody (data not shown) or the rabbit anti-rELI025 antibodies (**Figure 2.1-4B**).

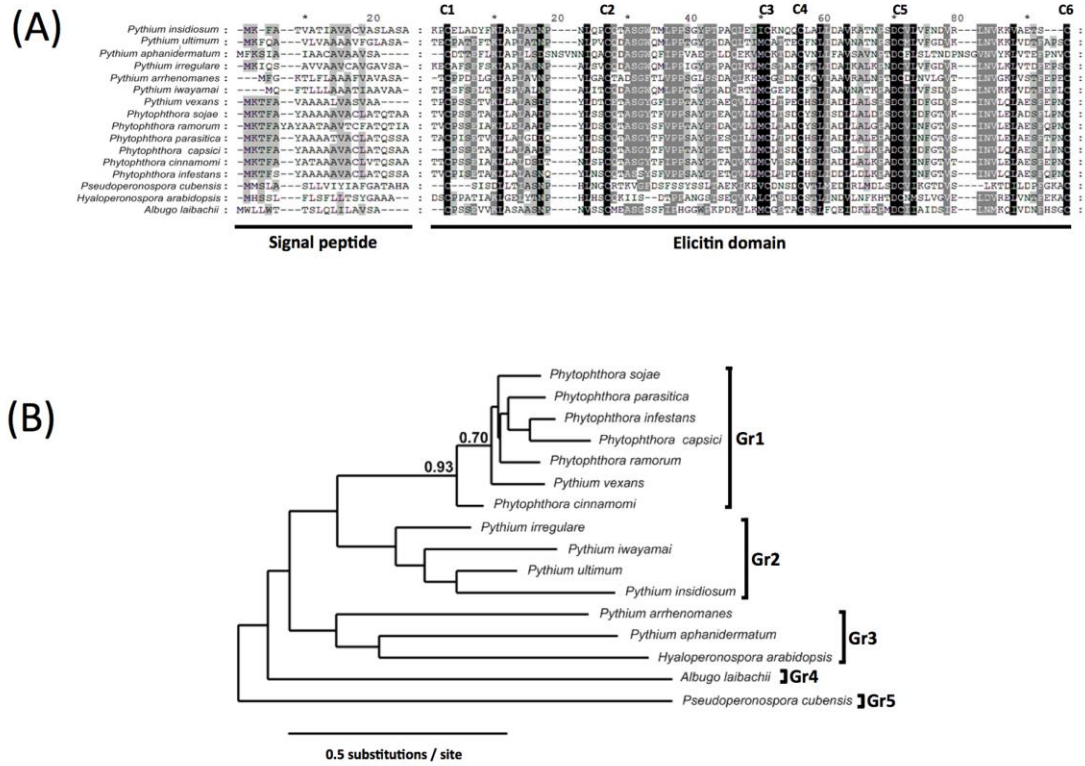

Figure 2.1-3. Sequence alignment and phylogenetic analysis of elicitin proteins: (**A**) Signal peptide (length, 17-23 amino acids) and elicitin domain (length, 83-94 amino acids) sequences of *P. insidiosum* ELI025 and the top BLAST hit proteins of 15 other oomycetes (**Table 2.1-1**) were aligned and compared. C1-C6 indicate conserved cysteine residues; (**B**) Phylogenetic analysis of elicitins by the neighbor-joining method. The phylogenetic tree, constructed from elicitin domain sequences of *P. insidiosum* ELI025 and the top BLAST hit proteins of 15 other oomycetes (**Table 2.1-1**), shows three major clades (as indicated by Gr1, Gr2 and Gr3; containing multiple sequences per clade) and two minor clades (as indicated by Gr4 and Gr5; containing one sequence per clade). Only the branch support values of 70 % or more are shown at corresponding nodes.

Gel-separated total proteins from crude extracts or supernatants of three *P. insidiosum* strains, had molecular weights ranging from 6 to 115 kDa (**Figure 2.1-4A**). In Western Blots, rabbit anti-rELI025 serum reacted only with the 10- and 15-kDa bands in CFA (culture filtrate antigen), which contains secreted proteins of *P. insidiosum* (**Figure 2.1-4B**). The rabbit anti-rELI025 serum did not react any proteins in SABH (soluble antigens from broken hyphae), which contains intracellular proteins (**Figure 2.1-4B**). The rabbit pre-immune serum did not detect any proteins in SABH or CFA. If the rabbit anti-rELI025 serum is pre-absorbed with rELI025 protein prior to Western Blot detection, the band intensities for the 10- and 15-kDa proteins were reduced by ~85% (data not shown).

The native ELI025 (nELI025) in CFA was treated with protein deglycosylases to remove either N- or O-linked glycosyl adducts. The SDS-PAGE and Western blot profiles (probed with the rabbit anti-rELI025 serum) show that the 15-kDa band disappears in the CFA treated with the N-linked deglycosylation enzyme (either alone or in combination with the O-linked deglycosylase; **Figure 2.1-5**). In contrast, the 10- and 15-kDa bands were both present in CFA treated with O-linked deglycosylase or in the no enzyme control.

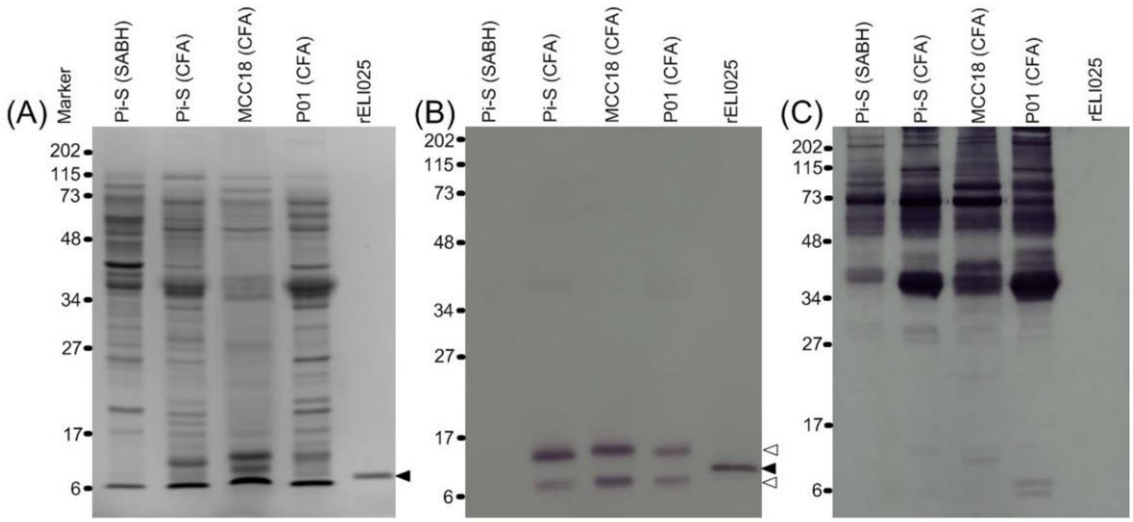

Figure 2.1-4. Immunoreactivity of the recombinant protein rELI025 and crude protein extracts of *P. insidiosum*. Crude proteins (i.e., SABH and CFA) extracted from three different strains of *P. insidiosum* (Pi-S, MCC18, and P01) and rELI025 are separated in a SDS-PAGE gel **(A)**. The separated proteins are analyzed by Western blot, using the rabbit anti-rELI025 antibodies **(B)**, or sera from patients with pythiosis **(C)**, as probe. The black arrow head indicates the 12.4 kDa band of rELI025. The white arrow heads indicate the 10- and 15-kDa bands of native ELI025. The numbers represent protein molecular weights standards, in kDa. (Abbreviations: SDS-PAGE, Sodium dodecyl sulfate polyacrylamide gel electrophoresis; CFA, culture filtrate antigen; SABH, soluble antigen from broken hyphae; rELI025, recombinant ELI025).

The 10- and 15-kDa bands excised from SDS-PAGE gel (**Figure 2.1-3A**) and Western blot membrane (**Figure 2.1-3B**) were analyzed by LC-MS/MS (see Methods). MASCOT analysis of MS data showed that the 10-kDa SDS-PAGE band-derived peptides with mass-to-charge ratio (m/z) of 569.6, 686.9 and 853.9, and the 15-kDa SDS-PAGE band-derived peptides with m/z of 458.3, 569.6, 686.9 and 853.9, matched the ELI025 protein in the *P. insidiosum*'s proteome (**Figure 2.1-6A**; **Table 2.1-2**). No peptide mass of the 10- and 15-kDa band excised from Western blots matched ELI025. Further MASCOT analyses of MS/MS data of the 10- and 15-kDa SDS-PAGE band-derived 686.9 peak, showed that the corresponding peptides had nearly-identical spectra (**Figure 2.1-6B**), and matched the same peptide sequence (KNQQCLALLDAVKA) predicted for ELI025. Similarly, MASCOT analyses of MS/MS data of the 10- and 15-kDa SDS-PAGE band-derived 853.9 peak, revealed that the corresponding peptides had nearly-identical spectra (data not shown), and matched another peptide sequence (KATNPSDCVLVFNDVRL) predicted for ELI025.

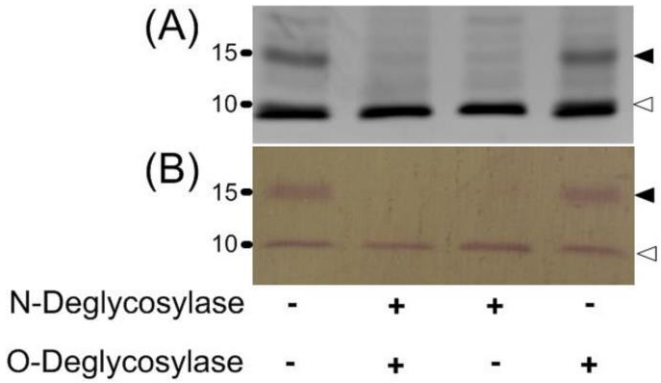

Figure 2.1-5. Protein deglycosylation of ELI025. CFA proteins were untreated (control; Lane 1) or treated with either N-Deglycosylase (N-glycosidase F; Lane 3), or O-Deglycosylase (a mixture of α-2-3,6,8,9-neuraminidase, endo-α-N-acetylgalactosaminidase, β-1,4-galactosidase, and β-N-acetylglucosaminidase; Lane 4), or both N- and O-Deglycosylases (Lane 2). The enzyme-treated proteins were separated on a SDS-PAGE gel **(A)**, and were further analyzed by Western blot, using the rabbit anti-rELI025 antibodies as primary antibody **(B)**. Only the low molecular weight portion of the gel and blot are shown. The black and white arrow heads point out the 15-kDa and 10-kDa bands. (Abbreviations: SDS-PAGE, Sodium dodecyl sulfate polyacrylamide gel electrophoresis; CFA, culture filtrateantigen; rELI025, recombinant ELI025).

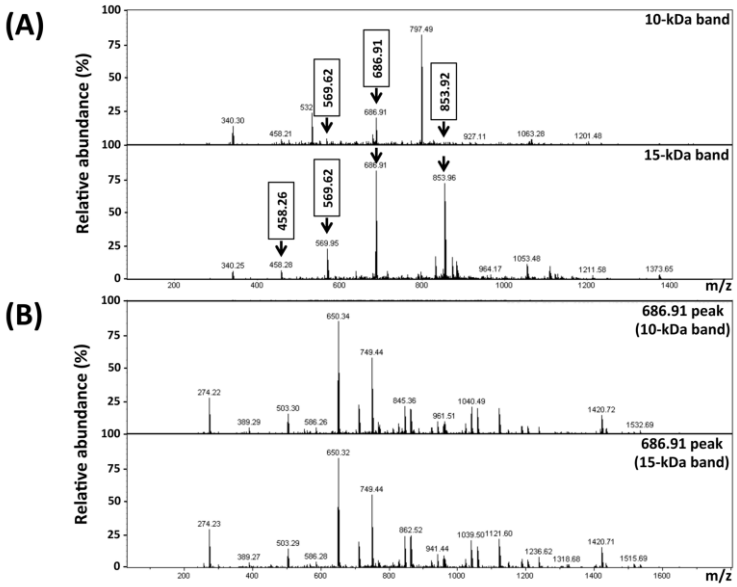

Figure 2.1-6. Mass spectrometric analyses of ELI025 by LC-MS/MS: (**A**) MS spectra of the 10- and 15-kDa SDS-PAGE band-derived proteins. The arrows indicate peptides with the mass-to-charge ratio (m/z), including 458.3, 569.6, 686.9 and 853.9, that match the ELI025 protein. Peptide sequences corresponding to the ELI025-matched peaks are shown in **Table 2.1-2**; (**B**) MS/MS spectra of the 686.91 peaks from the 10- and 15-kDa SDS-PAGE band-derived proteins.

Table 2.1-2. Mass spectrometric analyses of the 10- and 15-kDa SDS-PAGE band-derived proteins showing mass-to-charge ratio (m/z), average mass of peptide (M_r; calculated by MASCOT software), peptide sequences (identified by MASCOT software), BLAST search result (against ~15,000 genome-derived predicted proteins of *P. insidiosum*), and amino acid position of identified peptides.

SDS-PAGE band	m/z	M_r	Peptide sequence	BLAST search result	Amino acid position
10-kDa	569.62	1705.81	KATNPSDCVLVFNDVRL	ELI025	84-100
10-kDa	686.91	1371.72	KNQQCLALLDAVKA	ELI025	72-85
10-kDa	853.92	1705.81	KATNPSDCVLVFNDVRL	ELI025	84-100
15-kDa	458.26	1371.72	KNQQCLALLDAVKA	ELI025	72-85
15-kDa	569.62	1705.81	KATNPSDCVLVFNDVRL	ELI025	84-100
15-kDa	686.91	1371.72	KNQQCLALLDAVKA	ELI025	72-85
15-kDa	853.92	1705.81	KATNPSDCVLVFNDVRL	ELI025	84-100

Three serum samples each from pythiosis patients and normal individuals (control) were used as primary antibodies in Western blot to detect rELI025 or nELI025 in SABH and CFA. All control sera did not detect any proteins in SABH and CFA (data not shown). While pythiosis sera detected relatively-high molecular weight proteins of SABHs and CFAs (> 30 kDa), they failed to detect many lower molecular weight proteins, including the 10- and 15-kDa (representing nELI025). The patient sera also failed to react with the 12.4-kDa rELI025 band (**Figure 2.1-4C**).

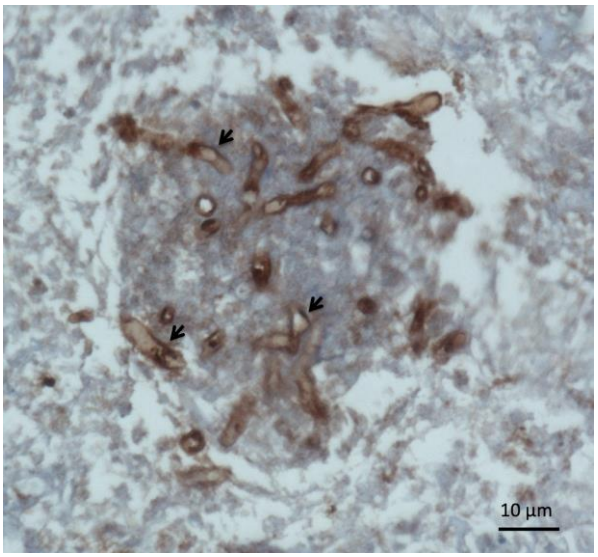

Figure 2.1-7. Cellular location of ELI025. Infected arterial tissue from a pythiosis patient was sequentially stained with rabbit anti-rELI025 serum, as the primary antibody, and then mouse anti-rabbit IgG antibody conjugated with horseradish-peroxidase, as the secondary antibody (see Materials and Methods). Images of the hyphae and location of ELI025 (indicated by arrows) were captured with a bright-field microscope. The scale bar represents 10 µm.

An immunohistochemical staining assay, using the rabbit anti-rELI025 serum, was used to target cellular localization of the *P. insidiosum* nELI025 in an infected arterial tissue. nELI025 markedly localized at

the cell surface and surrounding areas (**Figure 2.1-7**). No signal was detected with the rabbit pre-immune serum.

2.1.4 Discussion

The elicitin domain of ELI025 was predicted to contain three disulfide bonds (**Figure 2.1-1B**), which is a crucial characteristic of elicitin [34]. The *E. coli* strain rosetta-gami2 (DE3) was used to express ELI025 based on its reported facility in proper disulfide bond formation. The rabbit anti-rELI025 antibody, detected two proteins in CFA (10- and 15-kDa), but not in SABH (**Figure 2.1-4B**). The rabbit anti-rELI025 serum, pre-absorbed with rELI025, failed to effectively detect any proteins in CFA, indicating that the rabbit anti-rELI025 antibodies were specific to ELI025. The 10- and 15-kDa bands could be different proteins (i.e., other elicitins) or different isoforms of the same protein (ELI025 contains several predicted glycosylation linkages). Deglycosylation of CFA proteins indicated that the 10-kDa band represents nELI025 without glycosylation, while the 15-kDa band represents nELI025 with predominant N-linked glycosylation (**Figure 2.1-5**). Thus, nELI025 is a secreted glycoprotein, with two isoforms. The slightly-larger size of rELI025 (12.4 kDa) compared to the non-glycosylated nELI025 (10 kDa) is expected based on its expression in *E. coli* as a fusion with Thrombin and a His tag (**Figure 2.1-1A**).

Mass spectrometric analyses were used to confirm the identity of ELI025 in the 10- and 15-kDa bands of SDS-PAGE gel (**Figure 2.1-4A**). The sequences, determined by MS and MS/MS analyses, of the peptide mass 686.9 (KNQQCLALLDAVKA) and 853.9 (KATNPSDCVLVFNDVRL) of either the 10- or 15-kDa SDS-PAGE bands matched perfectly with the ELI025 predicted protein sequence. However, no peptide masses matching ELI025 were detected in the 10- and 15-kDa Western blot bands. This may result from the Western blot bands being contaminated with blocking reagent, primary antibody, secondary antibody, enzyme, and substrate, which may compromise detection sensitivity. As an alternative method to determine the identity of the 10- and 15-kDa bands in PVDF membrane, we used rabbit anti-ELI025 antibodies against rELI025 in Western blot analysis. The rabbit anti-rELI025 antibodies reacted only with the 10- and 15-kDa bands (**Figure 2.1-4B**), suggesting that the protein detected in the Western blots is ELI025.

Until recently, there were few genetic and molecular studies done in *P. insidiosum*, and to date, there is no transformation system for introducing foreign or modified *P. insidiosum* genes into the organism. In other oomycetes, transformations systems have been developed, and some of these depend on the *hsp70* and *ham34* promoters from the oomycete *Bremia lactucae* for transgene expression [78-81]. Since the upstream region of *ELI025* has conserved sequence found in the core promoter elements of many oomycete genes including *hsp70* and *ham34* (**Figure 2.1-2**), it may be possible to use already-developed transformation vectors such as pTH210, pHAMT34H, pHAMT35N/SK, and pHAMT35G [79], which utilize the *hsp70* and *ham34* promoters, for developing transformations systems in *P. insidiosum*. In addition, since *ELI025* is highly expressed, its upstream region could be used as a driving promoter for DNA transformation in *P. insidiosum*.

Elicitins form a unique group of proteins that have been found previously in two oomycete genera (*Phytophthora* spp. and some *Pythium* spp.), but not in fungi or bacteria [22,82,83]. In this study, we performed a similarity search of elicitin domain-containing proteins in the publicly-available genome, transcriptome, and proteome databases of various oomycetes (**Table 2.1-1**). In addition to *Phytophthora* and *Pythium* species, elicitin homologs were also found in oomycete genera *Pseudoperonospora*, *Hyaloperonospora*, and *Albugo*

(**Table 2.1-1**). As expected, phylogenetic analysis grouped the top BLAST hit elicitins of these oomycetes according to their genera based on previous classifications: *Phytophthora* species in Gr1 (with an exception for *P. vexans*), *Pythium* species in Gr2 and Gr3 (with an exception for *H. arabidopsis*), *Albugo* species in Gr4, and *Pseudoperonospora* species in Gr5 (**Figure 2.1-3B**). The conserved homology of elicitins among the closely related species also extended to both their core promoter sequences (**Figure 2.1-2**) and their signal sequences (**Figure 2.1-3A**). It should be noted that elicitins found thus far are in the more closely-related subgroups Pythiales, Peronosporales, and Albuginales. In contrast, no elicitin homologs were detected in *Aphanomyces* and *Saprolegnia* species, which belong to Saprolegnales, a more distantly-related oomycete lineage. This finding suggests that the origin and expansion of elicitins occurred after splitting off the oomycetes from its ancient progenitor and between the Saprolegnales lineage, and the ancestor lineage of the Pythiales, Peronosporales, and Albuginales.

Based on an extensive genome search (**Table 2.1-1**), elicitins are found in many oomycetes, but absent in all non-oomycete organisms, such as, fungi. Thus, elicitins are a signature character of the oomycetes. Among oomycetes, *P. insidiosum* is a notorious human pathogen. It shares microscopic features with some pathogenic fungi (such as, *Aspergillus* species, *Fusarium* species, and Zygomycetes). This can lead to misdiagnosis of pythiosis as a fungal infection [46,84], and results in delayed and improper treatment of patients. Because of the uniqueness of the elicitins to *P. insidiosum* among human pathogens, detection of *ELI025* or its gene product could aid in the development of more specific diagnostic tests for pythiosis, such as using the anti-rELI025 antibodies to detect *P. insidiosum* in infected tissue.

The detection of ELI025 in CFA, together with the predicted amino acid sequence harboring a signal peptide, indicate that ELI025 is a secreted protein. Additionally, the immunohistochemical staining assay of the infected tissue from a pythiosis patient showed localization of ELI025 at *P. insidiosum*'s cell surface and surrounding areas (**Figure 2.1-7**). This evidence suggests that, in *P. insidiosum*, ELI025 is expressed and secreted, both during *in vitro* growth and during infection of host tissue. Elicitins, secreted by the plant-pathogenic oomycetes, are beneficial to the pathogens by effecting host response and triggering programed cell death [23,24]. The role of elicitin secreted by *P. insidiosum* in humans is unknown. *Pythium* species are thought to be sterol auxotrophic microorganisms [22,32,33]. Like the elicitins from the plant-pathogenic oomycetes, *P. insidiosum* ELI025 has been predicted to contain a hydrophobic cavity that can bind a sterol molecule, implying that it can function as a sterol-carrying protein [31,34,85-88]. Western blot assays showed that the small proteins (< 30 kDa) in CFA, including nELI025, were not recognized by sera from patients with pythiosis (**Figure 2.1-4C**). Poor immunogenicity could prevent the elimination of ELI025 by host antibody responses, and therefore, it could allow ELI025 to act in sterol acquisition inside host tissue.

In conclusion, ELI025 has been successfully cloned and expressed in *E. coli*. Genetic, biochemical, and immunological characterization showed that ELI025 is a small glycoprotein, abundantly secreted by *P. insidiosum*. ELI025 had two isoforms (glycosylated and non-glycosylated form), and was not recognized by host antibodies. The upstream region of *ELI025* shared core promoter elements with the promoters of other oomycete genes. Among human fungal and oomycete pathogens, ELI025 is unique to *P. insidiosum*, and therefore, it is a potential target for development of more specific diagnostic tests. Characterization of ELI025 provided a new insight into the biology and pathogenesis of the understudied microorganism, *P. insidiosum*, and it could lead to a discovery of a new strategy for infection control.

2.1.5 References

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2.2 Biological variation of the effector protein, ELI025, of *Pythium insidiosum*

2.2.1 Introduction

Oomycetes are fungus-like microorganisms that genetically, biochemically, and physiologically differ from other eukaryotes, including fungi and parasites (Beakes et al., 2012; Kamoun, 2003; Mendoza et al., 1993). While most pathogenic oomycetes infect plants and some infect animals, the understudied oomycete *Pythium insidiosum* is capable of infecting humans and other animals, and causes the life threatening infectious disease, called pythiosis (De Cock et al., 1987; Kamoun, 2003; Mendoza and Vilela, 2013). *P. insidiosum* inhabits tropical and subtropical areas of the world (Gaastra et al., 2010; Mendoza and Vilela, 2013). Phylogenetic analyses divide *P. insidiosum* from different geographic origins into 3 groups: Clade-I (strains from Americas), Clade-II (strains from Asia and Australia continents), and Clade-III (strains from mostly Thailand) (Chaiprasert et al., 2010; Schurko et al., 2003). Almost all cases of pythiosis in humans have been reported from Thailand, whereas cases of pythiosis in animals have been found worldwide (Gaastra et al., 2010; Krajaejun et al., 2006; Mendoza, 2008; Mendoza and Vilela, 2009). No effective antimicrobial drug is currently available for treatment of *P. insidiosum* infection (Krajaejun et al., 2006; Permpalung et al., 2015). Extensive surgical removal of the infected organ (eye or leg) is an only treatment option for controlling the disease. Many patients with advanced infection die. Prompt and effective treatment could reduce the morbidity and mortality rates of pythiosis. Better understanding the basic biology of *P. insidiosum* is essential for developing effective infection controls.

Elicitins form a unique group of proteins that are present only in oomycetes (especially *Pythium* and *Phytophthora* species), but absent in all other organisms (Gaulin et al., 2010; Jiang et al., 2006; Madoui et al., 2009). In plant-pathogenic oomycetes, elicitins are involved in multiple biological and pathological processes, such as, acquisition of exogenous sterols (oomycetes are sterol auxotrophs), stimulation of host innate immunity (elicitins can function as a pathogen-associated molecular pattern molecules), and induction of host tissue necrosis (Boissy et al., 1996; Boissy et al., 1999; Kamoun et al., 1998; Mikes et al., 1998; Mikes et al., 1997; Nurnberger et al., 2004; Osman et al., 2001; Qutob et al., 2003; Yu, 1995). Recently, we have identified and characterized the elicitin-like glycoprotein, ELI025, from *P. insidiosum* (Lerksuthirat et al., 2015). *P. insidiosum* secrets large amount of glycosylated (15 kDa in size) and non-glycosylated (10 kDa) ELI025. ELI025 is abundant at the site of *P. insidiosum* infection. Its expression is up-regulated, upon exposure to body temperature. Failed recognition by host antibodies could prevent elimination of ELI025, and lead to ELI025 persistence during infection.

In this study, we characterize and compare the ELI025 protein secreted by 24 different strains of *P. insidiosum* from various isolation sources (i.e., humans, animals, and environment) and phylogenetic origins (i.e., Clade-I, -II, and -III). The information obtained could lead to appropriate clinical applications of this unique protein for the diagnosis and/or treatment of pythiosis.

2.2.2 Materials and methods

Strains, growth condition, and antigen preparation: Twenty-four strains of *P. insidiosum* isolated from patients with pythiosis (n=14), animals with pythiosis (n=8), environment (n=1), and unknown source of isolation (n=1) were used in this study (**Table 2.2-1**). The organisms were maintained on Sabouraud dextrose (SD) agar at room temperature, and subcultured once a month, until use. To prepare hyphal material for crude

protein extraction, several small pieces of SD agar with actively-growing *P. insidiosum* colony were transferred to a flask with 100 ml SD broth, and incubated with shaking at 37 °C for 7 days. After thimerosol [0.02% (wt/vol); Sigma] was added to the culture, the hyphae were filtered through a Durapore membrane filter (0.22-μm pore size; Millipore) and used for genomic DNA extraction (see below). Cell-free broth cultures, from each of the *P. insidiosum* strains Pi05, Pi07, Pi09, Pi11, Pi20, Pi35, Pi44, Pi45 and Pi49 (**Table 2.2-1**), were used to prepare culture filtrate antigens or CFA (representing secreted proteins), using the protocol described by Krajaejun et al. (2002). Protein concentration was determined using a Biorad Bradford assay kit (Bradford, 1976). CFA was stored at -20 °C until use.

Table 2.2-1: Twenty-four *P. insidiosum* strains used in this study, and corresponding information, including strain ID, reference number, source of isolation, phylogenetic clade, presence (+) or absence (-) of PCR product amplified by the primer Pair#1 and Pair#2, and nucleotide accession number for *ELI025* and rDNA sequences.

Strain ID	Reference number	Source of isolation (country)	rDNA-based phylogenetic clade	PCR product		Accession number	
				Pair#1	Pair#2	ELI025	rDNA
Pi02	CBS579.85	Equine (Costa Rica)	Clade-I	+	-	-	AB971176
Pi03	CBS577.85	Equine (Costa Rica)	Clade-I	+	-	-	AB971177
Pi04	CBS576.85	Equine (Costa Rica)	Clade-I	+	-	-	AB898106
Pi05	CBS575.85	Equine (Costa Rica)	Clade-I	+	-	AB971194	AB971178
Pi06	CBS574.85	Equine (Costa Rica)	Clade-I	+	-	-	AB971179
Pi07	CBS573.85	Equine (Costa Rica)	Clade-I	+	-	AB971195	AB971180
Pi08	CBS580.85	Equine (Costa Rica)	Clade-I	+	-	-	AB898107
Pi09	CBS101555	Equine (Brazil)	Clade-I	+	-	AB971196	AB971181
Pi11	BL	Human/Artery (Thailand)	Clade-II	+	+	AB898692	AB898109
Pi15	SIMI8727	Human/Artery (Thailand)	Clade-II	+	+	-	AB898111
Pi16	CBS119452	Human/Artery (Thailand)	Clade-II	+	+	-	AB971182
Pi20	CBS119455	Human/Eye (Thailand)	Clade-II	+	+	AB898694	AB971183
Pi23	MCC10	Human/Disseminated (Thailand)	Clade-II	+	+	-	AB898115
Pi26	SIMI4523-45	Human/Eye (Thailand)	Clade-II	+	+	-	AB898117
Pi35	Pi-S	Human/Artery (Thailand)	Clade-II	+	+	FX528334	AB898124
Pi42	CR02	Environment (Thailand)	Clade-II	+	+	-	AB971184
Pi44	CBS119454	Human/Disseminated (Thailand)	Clade-III	-	-	-	AB971185
Pi45	MCC13	Human/Skin (Thailand)	Clade-III	-	-	-	AB971186
Pi46	SIMI3306-44	Human/Eye (Thailand)	Clade-III	-	-	-	AB971187
Pi47	SIMI2921-45	Human/Eye (Thailand)	Clade-III	-	-	-	AB971188
Pi48	SIMI4763	Human/Skin (Thailand)	Clade-III	-	-	-	AB971189
Pi49	SIMI7695-48	Human/Artery (Thailand)	Clade-III	+	-	AB898695	AB898127
Pi50	ATCC90586	Human/Skin (USA)	Clade-III	-	-	-	AB971190
Pi51	Pi51	Unknown/(Thailand)	Clade-III	-	-	-	AB898128

Serum samples: Seventeen serum samples from Thai patients with pythiosis were obtained for immunological characterization of the ELI025 protein. These patients were diagnosed by either culture identification (Chaiprasert et al., 1990) or detection of anti-*P. insidiosum* antibody (Imwidthaya and Srimuang, 1989; Jindayok et al., 2009; Krajaejun et al., 2009; Krajaejun et al., 2002; Vanittanakom et al., 2004). Control sera were obtained from healthy blood donors (Blood Bank Division, Department of Pathology, Ramathibodi Hospital; n=8), thalassemic patients (n=4), patients with positive antinuclear antibody (n=1) or rheumatoid factor (n=1), and patients with other infectious diseases (two each of aspergillosis and mucormycosis, and one each of histoplasmosis, candidiasis, cryptococcosis, human immunodeficiency virus infection, hepatitis B virus infection, and syphilis). All sera were kept at -20 °C until use.

Genomic DNA extraction: Genomic DNA (gDNA) was extracted from *P. insidiosum*, using the salt-extract protocol described by Lohnoo et al. (2014). Briefly, ~500 mg of hyphal mat was transferred to a 2-ml screw-cap tube, containing glass beads (diameter, 710-1,180 μm; Sigma) and 400 μl salt homogenizing buffer [0.4 M NaCl, 10 mM Tris–HCl (pH 8.0), 2 mM EDTA (pH 8.0)], and homogenized using a Qiagen TissueLyzer MM301 machine (setting: 30 Hz for 2 min). After SDS (final concentration, 2%) and proteinase K (final concentration, 400 μg/ml) were added, the cell lysate was incubated at 55 ºC for an hour, 300 μl 6 M NaCl was added, and the solution was vigorously vortexed for 30 s, and centrifuged (10,000 x g) at room temperature for 30 min. To precipitate the gDNA, the resulting supernatant was mixed with an equal volume of isopropanol and held at -20 ºC for 1 hr. The pellet was collected by centrifugation, washed with 70% ethanol, air dried, and resuspended in 100 μl TE buffer (pH 8.0). The extracted gDNA was stored at -20ºC until use.

SDS-PAGE and Western blot analysis: CFA proteins were separated by SDS-PAGE (4% stacking and 12% separating gels) using a Biorad Mini-PROTEAN II apparatus (setting: 150 V for 65 min). The SDS-PAGE gel was stained with Coomassie blue R-250. Pre-stained broad range protein markers (Biorad) were used to estimate molecular weights of the separated proteins. Proteins were blotted onto a PVDF membrane (Merck Millipore), using a Biorad Mini Trans-Blot cell apparatus (setting: 100 V for 60 min). The blotted membrane was blocked with 5% skim milk (Sigma) in TBS [150 mM NaCl, 10 mM Tris-Cl (pH 7.5)], and washed 3 times with TTBS [500 mM NaCl, 20 mM Tris-Cl (pH 7.5), 0.05% (v/v) Tween-20]. The membrane was incubated with the rabbit anti-ELI025 antibody (1:2,000 in the blocking buffer) (Lerksuthirat et al., 2015) for 2 hr at room temperature, and washed 3 times with TTBS. Goat anti-rabbit IgG, conjugated with alkaline phosphatase (Southern Biotech; 1:5,000 in the blocking buffer), was added to the membrane, incubated for 2 hr at room temperature, and washed 3 times with TTBS. Western blot signals were developed using BCIP and NBT.

ELISA: A 96-well polystyrene plate (Corning) was coated (100 μl/well) overnight at 4 ºC with either 5 μg/ml of recombinant ELI025 [rELI025; generated by Lerksuthirat et al. (2015)] in 0.1 M sodium phosphate buffer (pH 7.4) or 5 μg/ml of CFA [prepared from the *P. insidiosum* strain Pi35] in 0.1 M carbonate buffer (pH 9.6) and 1.5% NaCl. Unbound proteins were removed by washing 4 times with TPBS pH 7.4 (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.76 mM KH_2PO_4 and 0.05% Tween-20). Each well was blocked with 250 μl of 0.5% (w/v) BSA (Merck) in 0.1 M sodium phosphate buffer (pH 7.4) at 37 ºC for 1 hr, and washed 4 times with TPBS. Serum samples diluted in PBS pH 7.4 (1:800 for ELI025; 1;1,600 for CFA) were added to each well (100 μl/well), and incubated at 37ºC for 1 hr. The plate was washed 4 times with TPBS. The goat anti-human

IgG conjugated with peroxidase (Jackson Immuno Research; 1:100,000 in PBS pH 7.4) was added to each well (100 µl/well), and incubated at 37 ºC for 1 hr. After the plate was washed 4 times with TPBS (pH 7.4), color signal was developed using an ELISA substrate kit (Biorad). The reaction was stopped by adding 0.3 N sulfuric acid. ELISA signal was measured at OD_{450} using an Infinite 200Pro microplate reader (Tecan). ELISA signals were analyzed by the GraphPad Prism program version 5 (GraphPad Software, USA). Statistical difference between two sets of ELISA signals was determined by two-tailed unpaired t-test.

Polymerase chain reaction: All PCR amplifications were carried out in a Mastercycler Nexus thermal cycler (Eppendorf). Two independent pairs of primers were used to amplify portions of the ELI025 sequence: Pair#1 [ELI025_F1 (5'-TACAACGAGACCAAGCCGTG-3') and ELI025_R1 (5'-GGCCTTGCAGCTCGTCTC-3')]; and Pair#2 [ELI025-full-F1 (5'-CACGCGGTGTTCGTTCCATG-3') and ELI025-full-R1 (5'-GCGTCAAGATGAGAAACGAGG-3')]. The primer Pair#1 (0.5 µM each primer) were included in a 20-µl PCR reaction containing 100 ng gDNA and the KAPA Tag PCR kit reagent (Kapa Biosystems, USA) (PCR condition: 35 cycles of 95 °C for 30 s, 60°C for 30 s, and 72°C for 40 sec). The primer Pair#2 (0.5 µM each primer) were included in a 50-µl PCR reaction containing 100 ng gDNA, 0.02 U/µl DNA polymerase (Phusion), 1x Phusion buffer, and 200 µM dNTPs (PCR condition: 98 °C for 30 s, 35 cycles of 98 °C for 10 s and 72 °C for 40 s, and 72°C for 10 min). The primer ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White et al., 1990) were used to amplified rDNA sequences in a 20-µl reaction, containing 100 ng gDNA, 0.025 U/µl Taq DNA Polymerase (Fermentas), 1x KCl buffer, and 200 µM dNTPs (PCR condition: 95 °C for 6 min, 35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min, and 72°C for 10 min). All PCR products were purified using a NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel) and assessed by 1% agarose gel electrophoresis.

DNA sequencing and nucleotide sequence accession numbers: The *ELI025* and rDNA PCR products were sequenced using the corresponding primers (Pair#1, Pair#2, or ITS1/4), and an ABI PRISM BigDye™ terminator cycle sequencing ready reaction kit, version 3.1 (Applied Biosystems, USA). Automated sequencing was performed in an ABI 3100 Genetic Analyzer, using the Applied Biosystems Sequencing software (Applied Biosystems, USA). All *ELI025* and rDNA sequences obtained in this study have been submitted to DNA data bank of Japan (DDBJ), under the accession numbers shown in **Table 2.2-1**.

Bioinformatic and phylogenetic analyses: *ELI025* coding and deduced amino acid sequences were aligned and analyzed using ClustalX (Larkin et al., 2007) and GeneDoc (Nicholas et al., 1997). Protein domains were identified using the NCBI conserve domain search (Marchler-Bauer et al., 2015). O- and N-linked glycosylation sites were predicted online at the NetNGlyc 1.0 and NetOGlyc 4.0 servers (www.cbs.dtu.dk/services/) (Julenius et al., 2005).

rDNA sequences from 24 strains of *P. insidiosum* (**Table 2.2-1**) were subjected to phylogenetic tree construction, using the online program at http://www.phylogeny.fr/ (Dereeper et al., 2008). All sequences were aligned by MUSCLE (Edgar, 2004), phylogenetically analyzed by the Neighbor-Joining algorithm with 1,000 bootstraps (Saitou and Nei, 1987) and the Kimura 2 parameters substitution model (Kimura, 1980). Trees were constructed using TreeDyn (Chevenet et al., 2006). rDNA sequences from *Pythium aphanidermatum* (Accession number: AY151180), *Pythium deliense* (AY151181), *Pythium grandisporangium* (AY151182), and *Lagenidium giganteum* (AY151183) were included as outgroups.

2.2.3 Results

Phylogenetic analysis of *P. insidiosum*: rDNA-based Neighbor-Joining phylogenetic analysis classified 24 *P. insidiosum* strains into 3 clades: Clade-I, -II, and -III (**Table 2.2-1**; **Figure 2.2-1**). Clade-I contained strains from the Americas [Costa Rica (n=7) and Brazil (n=1)]. All 15 Thai strains were grouped in Clade-II (n=8) and -III (n=7). One strain isolated from a pythiosis patient living in USA was placed in Clade-III. Clade-II was more closely related to Clade-I than to Clade-III. All *P. insidiosum* strains formed a group that is distinct from the outgroup species (i.e., other *Pythium* species and *L. giganteum*; **Figure 2.2-1**).

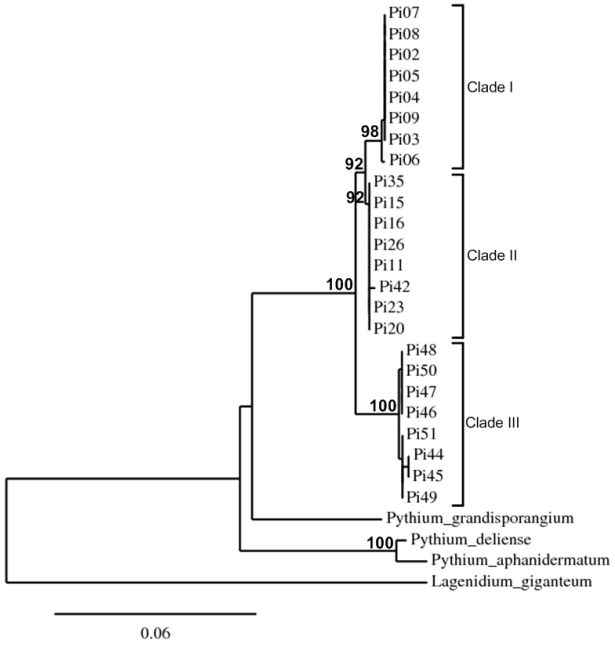

Figure 2.2-1. Phylogenetic analysis of *P. insidiosum* strains: rDNA sequences from 24 strains of *P. insidiosum* (**Table 2.2-1**) and 4 outgroup oomycetes, including *Pythium aphanidermatum* (accession number, AY151180), *Pythium deliense* (AY151181), *Pythium grandisporangium* (AY151182), and *Lagenidium giganteum* (AY151183), are used for Neighbor-Joining based phylogenetic analysis. The reliability of the inferred trees was tested using 1,000 bootstraps. The branch support values of at least 70% are shown. The scale bar at the bottom refers to the rate of nucleic acid substitution.

Genetic and biochemical variations of ELI025: Two sets of primers (Pair#1: ELI025_F1 and ELI025_R1; Pair#2: ELI025-full-F1 and ELI025-full-R1) were designed to amplify *ELI025* coding and flanking sequences from gDNAs of 24 *P. insidiosum* strains (**Table 2.2-1; Figure 2.2-2A**). Pair#1 amplified a ~280 bp amplicon from all strains in Clade-I and -II, and one strain (Pi49) in Clade-III (**Figure 2.2-2B**). Pair#1 produced multiple faint bands in most of Clade-III strains (**Figure 2.2-2B**). Pair#1-derived PCR products of Clade-II strains were more prominent than that of Clade-I and -III strains. Pair#2 amplified an intense band (with slight size variation among the strains, ranging from ~1,080 bp to ~1,120 bp) from all Clade-II strains, but did not amplify any PCR product from other strains (**Figure 2.2-2B**). The universal fungal rDNA primer, ITS1/4 (White et al., 1990), amplified the expected PCR product (~930 bp) from all strains (**Figure 2.2-2B**).

Pair#1-derived PCR products (*ELI025*-coding region; **Figure 2.2-2A** and **B**), amplified from the representative strains of Clade-I (Pi05, Pi07, and Pi09), Clade-II (Pi11, Pi20, and Pi35), and Clade-III (Pi49),

were sequenced and compared. Alignment of all *ELI025*-coding sequences (273-bp long) showed 93-99% identity, and 18 sites of single-nucleotide polymorphism (**Figure 2.2-3A**). Alignment of the corresponding translated 91-amino acid sequences revealed 97-100% identity between the strains, with three positions of amino acid polymorphisms: position 25 [Glutamate (E) for Clade-I strains; Glutamine (Q) for Clade-II and -III strains], position 29 [Lysine (K) for Clade-I strains; Threonine (T) for Clade-II and -III strains], and position 49 [Valine (V) for Clade-I strains; Isoleucine (I) for Clade-II and -III strains] (**Figure 2.2-3B**). Conserved cysteines were found in all strains and are predicted to form three internal disulfide bonds: cysteine position 7 and 71, 27 and 56, and 51 and 90 (**Figure 2.2-3B**, labels C1, C2 and C3) (**Figure 2.2-3B**). Three predicted O-linked glycosylation sites (position 29, 31 and 34) and two N-linked glycosylation sites (position 2 and 67) were present in all sequences (**Figure 2.2-3B**, labels O and N).

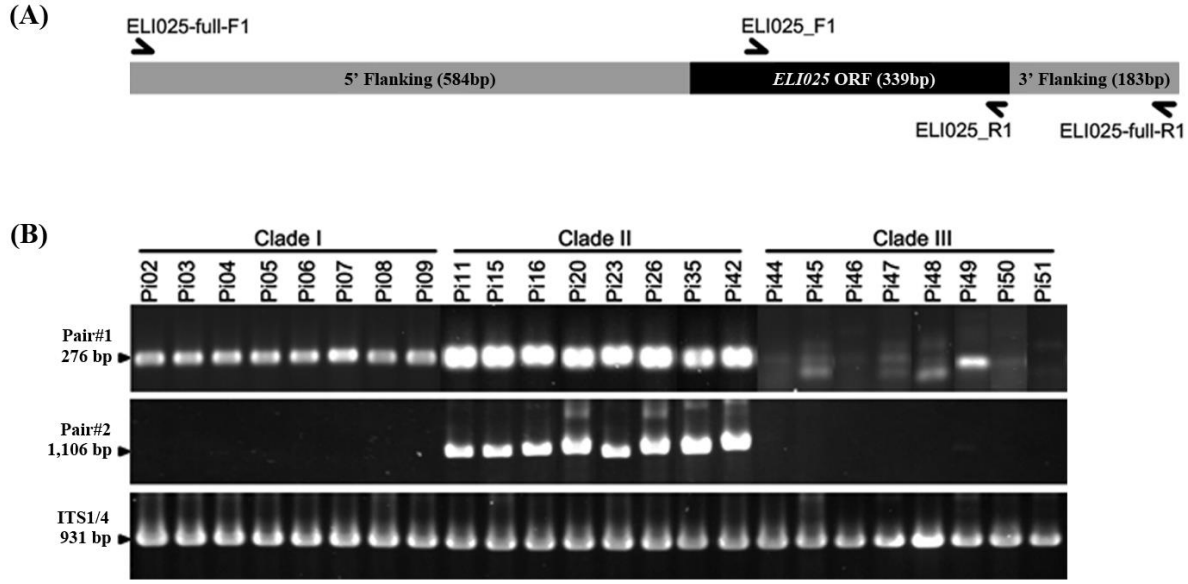

Figure 2.2-2. PCR amplification of *ELI205* and rDNA: (**A**) Structure of the *ELI025* open reading frame (ORF; labelled black) and flanking (labelled gray) sequences; Primer annealing sites are indicated: Pair#1 (ELI025_F1 and ELI025_R1) and Pair#2 (ELI025-full-F1 and ELI025-full-R1); (**B**) Agarose gel electrophoresis of PCR products amplified by primer Pair#1 (*ELI025* coding sequence; 276 bp), Pair#2 (*ELI025* coding and flanking sequences; 1,106 bp), and ITS1/ITS4 (rDNA sequences; 931 bp).

Western blot analysis of CFA (crude extract representing *P. insidiosum* secreted proteins) (**Figure 2.2-4A**) was performed using rabbit anti-ELI025 antibodies. Both 15- and 10-kDa proteins [which represent glycosylated and non-glycosylated forms of ELI025, respectively (Lerksuthirat et al., 2015)] were detected in the representative strains of Clade-I (Pi05, Pi07, Pi09) and Clade-II (Pi11, Pi20, Pi35) (**Figure 2.2-4B**). In contrast, only the 10-kDa protein (non-glycosylated ELI025) was detected in the representative strains of Clade-III (Pi44, Pi45, Pi49; **Figure 2.2-4B**). Lower amounts of the 10- and 15-kDa proteins were detected for strain Pi09, compared to the other strains (**Figure 2.2-4B**).

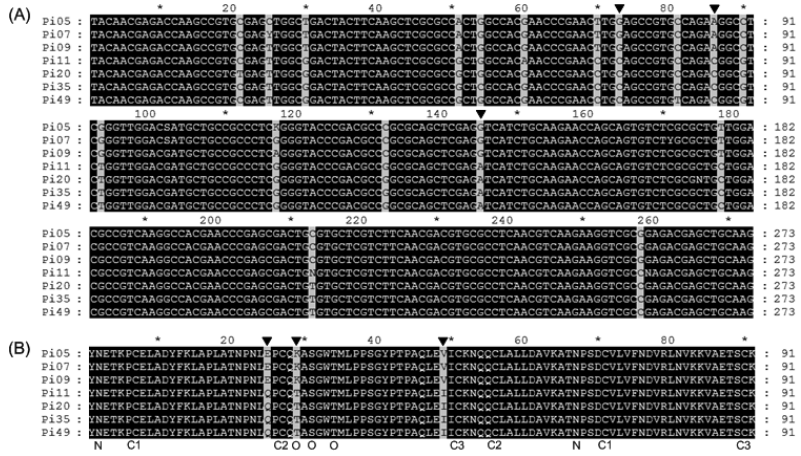

Figure 2.2-3. Sequence analyses of ELI025: (**A**) Alignment of the ELI025-coding sequences (273 bp) from 7 different *P. insidiosum* strains shows 18 sites (gray) of nucleotide polymorphism (arrow heads indicate nucleotide substitutions that result in amino acid changes); (**B**) Alignment of the *ELI025*-translated amino acid sequences (91 amino acids long) demonstrates 3 amino acid changes (arrow heads), 3 predicted disulfide bonds (C1, C2, C3), and 3 predicted O-linked glycosylation sites (O), and 2 predicted N-linked glycosylation sites (N).

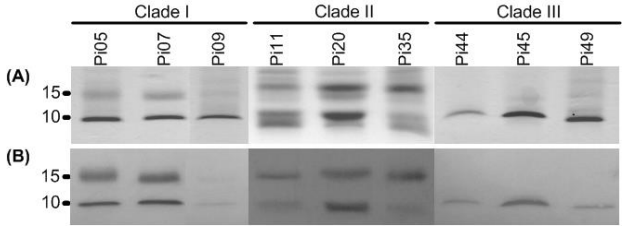

Figure 2.2-4. SDS-PAGE and Western blot analysis of *P. insidiosum* proteins: (**A**) Coomassie Blue stain of SDS-PAGE gel of culture filtrate antigen (CFA) prepared from the representative Clade-I, -II, and -III *P. insidiosum* strains; (**B**) Western blot analysis shows the separated CFAs, after probing with the rabbit anti-ELI025 antibody. Estimated protein molecular weights (10 and 15 kDa) are shown on the left.

Immunoreactivity of ELI025 against sera from pythiosis patients: ELISA assays were performed with rELI025-coated plates and sera from pythiosis patients (n=17) and controls (n=24). The mean ELISA signal for pythiosis sera was 8.2-fold higher than the signal for control sera [0.882 (SD, 0.573) vs. 0.108 (SD, 0.101); P < 0.0001; **Figure 2.2-5B**]. By comparison, ELISAs performed with CFA-coated plates had a mean ELISA signal for pythiosis patient sera which was 55.3-fold higher than that of control sera [1.382 (SD, 0.136) vs. 0.025 (SD, 0.016); P < 0.0001; **Figure 2.2-5A**].

A cut-off value [defined as the mean ELISA signal of control sera plus 3 SDs (Krajaejun et al., 2002)] was used to differentiate a positive test reaction from controls. All control sera had ELISA signals below the CFA- and rELI025-based cut-off values, indicating both assays had 100% detection specificity (**Figure 2.2-5A** and **B**). For the CFA-based ELISAs, all pythiosis patient sera had values above the cut-off (0.073) for a 100% detection sensitivity (**Figure 2.2-5A**). By contrast, only 12 of the 17 pythiosis patient sera had rELI025-based ELISA values above the cut-off (0.410) to give 71% detection sensitivity (**Figure 2.2-5B**).

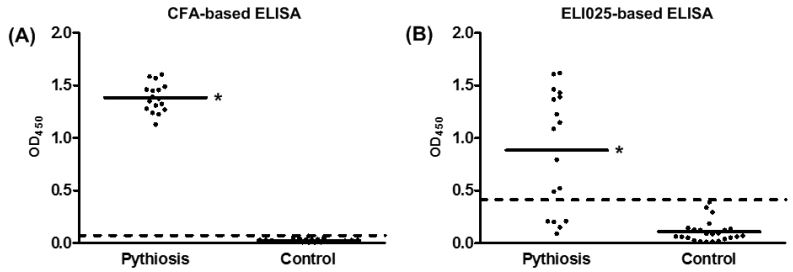

Figure 2.2-5. Immunoreactivity of CFA and ELI025 against pythiosis and control sera: (**A**) CFA-based ELISA shows that CFA is recognized by pythiosis sera (n=17), but not by control sera (n=24); (**B**) ELI025-based ELISA shows that ELI025 has variable immunoreactivity against pythiosis sera, and was minimally recognized by control sera. The solid line represents mean ELISA signal (measured at OD_{450}). Dash line shows ELISA cut-off value (calculated by mean ELISA signal of control sera plus 3 SDs). Asterisk indicates significant difference ($P < 0.0001$) between mean ELISA signals of pythiosis and control sera.

2.2.4 Discussion

Based on the rDNA sequences, all 24 *P. insidiosum* strains, used in this study (**Table 2.2-1**), can be classified into 3 phylogenetic groups, Clade-I, -II, and -III (**Figure 2.2-1**), which is consistent with previously reported phylogenetic classifications (Schurko et al., 2003; Chaiprasert et al., 2010). Fragments produced by PCR using primer Pair#1 and Pair#2, (targeting *ELI025*-coding and -flanking sequences of *P. insidiosum*; **Figure 2.2-2A**), were used to characterize the *ELI025* gene of clades-I, -II, and -III. The primer Pairs#1 and #2 successfully amplified product for 17 strains and 8 strains, respectively (**Figure 2.2-2B**), suggesting that the *ELI025* sequences are genetically variable, and the primer annealing sites of Pair#1 were more conserved than that of Pair#2. The patterns of positive and negative PCR reactions were clade-specific, and correlated with the rDNA sequence based phylogenetic groups: Pair#1(+)/Pair#2(-) for Clade-I strains, (+)/(+) for Clade-II strains, and (-)/(-) for Clade-III strains [with one exception for strain Pi49: (+)/(-)] (**Table 2.2-1**; **Figure 2.2-2B**). Thus, to a great extent, Pair#1- and Pair#2-derived PCR pattern could be used as a simple method for genotyping *P. insidiosum*, that could abrogate the necessity for rDNA sequence analyses. For example, the (+)/(+) pattern suggests Clade-II genotype, while the (-)/(-) pattern suggests Clade-III genotype. However, if the (+)/(-) pattern is observed, rDNA sequence analysis is required to discriminate Clade-I and Clade-III genotypes. Further study using a greater number of *P. insidiosum* strains is necessary for evaluation of this genotyping method.

All PCR patterns [(+)/(+), (-)/(-), or (+)/(-)] of the *ELI025* sequence were observed in the isolates from all human patients (who had different site of infection, i.e., eye, artery, skin), while only the (+)/(-) PCR pattern was observed in the isolates from animals (**Table 2.2-1**; **Figure 2.2-2B**). These findings suggest that the human hosts, regardless of clinical manifestations, were susceptible to *P. insidiosum* with all *ELI025*-based genotypes, while the animal hosts were susceptible to *P. insidiosum* with the (+)/(-) genotype.

Previously, we demonstrated that three different *P. insidiosum* strains, isolated from Thai patients with pythiosis, secreted two forms of ELI025: glycosylated (15 kDa) and non-glycosylated (10 kDa) (Lerksuthirat et al., 2015). As shown here, ELI025 was also secreted by all other *P. insidiosum* strains isolated from different sources (humans, animals, and environment) and from different phylogenetic groups (Clade-I, -II, and -III) (**Table 2.2-1**; **Figure 2.2-4)**. Nevertheless, Clade-III strains, which are phylogenetically distinct from Clade-I and -II strains (**Figure 2.2-1**), lacked the glycosylated ELI025 (15-kDa band; **Figure 2.2-4**). Sequence variations in

the *ELI025* gene (i.e., base changes, deletions, or insertions), which are likely responsible for different amplicon patterns (**Figure 2.2-2B**), may also lead to the lack of glycosylated ELI025 in Clade-III strains. To address this issue, the *ELI025*-coding sequences from the representative Clade-I, -II, and -III strains were aligned and analyzed (**Figure 2.2-3A**). All ELI025 protein sequences have predicted glycosylated sites at the amino acid positions 2 and 67 (for N-link glycosylation) and 29, 30 and 34 (for O-link glycosylation) (**Figure 2.2-3B**). Eighteen sites of nucleotide polymorphism were identified (**Figure 2.2-3A**), but only three sites (positions 25, 29, and 49) are associated with amino acid changes: E25-K29-V49 for Clade-I strains; and Q25-T29-I49 for both Clade-II and -III strains (**Figure 2.2-3B**). Strikingly, changes in the amino acid sequence of ELI025 cannot explain the lack of the glycosylated form in Clade-III strains, because the deduced amino acid sequences of ELI025 in the Clade-II and Clade-III strains are identical. Another alteration in the Clade-III strains, such as a defect in post-translational modification or glycoproteins secretion, must explain the failure to detect glycosylated ELI025.

The crude extract, CFA, comprises hundreds of *P. insidiosum* secreted proteins, including ELI025. CFA-based ELISA showed that all sera from 17 Thai patients with pythiosis, but not sera from the control group, had robust antibody responses against *P. insidiosum* (**Figure 2.2-5A**). In contrast, the rELI025-based ELISA had variable responses with the same set of pythiosis sera. While 70% of the assays had positive responses (above the cut-off), ~30% of these sera were indistinguishable from the controls (**Figure 2.2-5B**). Since Clade-I strains have never been isolated in Thailand, Thai patients that provided the sera were likely infected with a strain from either Clade-II or -III (**Table 2.2-1**). Host immunity might recognize and respond to each particular form of ELI025 differently. Selective production or secretion of glycosylated ELI025, observed among the Clade-II and -III strains (**Figure 2.2-4**), might contribute to the variable host antibody responses to ELI025 (**Figure 2.2-5B**). In the plant-pathogenic oomycete *Pythium vexans*, two secreted elicitins, Vex1 and Vex2, were identified (Huet et al., 1995). The glycosylated elicitin, Vex1, exhibits more robust toxicity in the plant host, compared to the non-glycosylated elicitin, Vex2, suggesting an important role of protein glycosylation in host response and pathogenesis.

In conclusion, ELI025, secreted by *P. insidiosum* from different phylogenetic groups, had different genetic, biochemical, and immunological characteristics. Selective production or secretion of glycosylated ELI025 by different *P. insidiosum* strains might contribute to variable host antibody responses, so that ELI025-based ELISAs would not be clinically relevant. ELI025 is a unique protein present in all strains of *P insidiosum* from different hosts and geographic origins. Therefore, direct detection of this pathogen via immunohistochemical staining of ELI025 could prove to be useful for diagnosis of *P. insidiosum* infection. In addition, based on the Clade-specific patterns of PCR outcomes using primer Pairs#1 and #2, a simple PCR test could be used in the clinic for genotyping *P. insidiosum*.

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2.3 Anti-effector antibody-based assay for histodiagnosis of pythiosis

2.3.1 Introduction

Early and accurate diagnosis is a key to prompt and effective treatment of pythiosis. The current diagnostic modalities, including culture identification (1–3), serodiagnosis (4–14) and molecular-based detection (14–19), are fraught with problems. For example, culture identification is time-consuming and often fails to grow and identify the organism. Serodiagnostic tests (i.e., immunodiffusion, ELISA, Western blot, hemagglutination, and immunochromatographic test), for detection of anti-*P. insidiosum* antibodies, usually produce false negative results for sera from patients with ocular pythiosis. Molecular assays, based on PCR and sequence homology, require skilled personnel and sophisticated equipment, not readily available in the endemic regions of pythiosis. In addition, limited yield or degradation of the extracted DNA compromises the diagnostic performance of such assays.

As alternatives, several investigators have developed immunohistochemical assays (IHCs) for the diagnosis of pythiosis. These assays are based on rabbit antiserum (as primary antibody), raised against *P. insidiosum* crude extracts [i.e., culture filtrate antigen (CFA), and soluble antigen from broken hyphae (SABH)] (20,21). IHC showed good detection sensitivity, but limited detection specificity, due to cross reactivity of the assay with some pathogenic fungi, i.e., *Fusarium* and *Conidiobolus* species (17,21). Therefore, specificity of the IHC assays needs to be improved.

Elicitins form a group of proteins found only in a phylogenetically-distinct group of microorganisms, the oomycetes, but absent in all other microorganisms, including true fungi (22–25). Recently, we reported a number of elicitins from the *P. insidiosum* transcriptome, and one of which, ELI025, is highly expressed and appears at the pathogen cell surface (25–27). Since the elicitins are unique to *P. insidiosum* among the human pathogens, direct detection of ELI025 could aid in the development of a more specific IHC for pythiosis. In this study, we developed a new IHC, using the rabbit anti-ELI025 antibody (anti-ELI) (25), for histodiagnosis of *P. insidiosum*, and compared its performance with the established IHC, using the rabbit anti-CFA antibody (anti-CFA) (21).

2.3.2 Methods

Paraffin-embedded histological sections: Eighty-seven paraffin-embedded samples were prepared from pure cultures of *P. insidiosum* or other fungi (defined as 'culture blocks'; **Table 2.3-1**) and infected tissues (defined as 'tissue blocks'; **Table 2.3-2**) for evaluation of IHC. Nineteen strains of *P. insidiosum* [**Table 2.3-1**; reference codes CP01-19; isolated from environment (n=2) and patients with vascular pythiosis (n=9), ocular pythiosis (n=4), cutaneous pythiosis (n=2), and other forms of pythiosis (n=2)], and 31 isolates of other fungi [**Table 2.3-1**; reference codes CC01-31; served as controls; including *Fusarium* spp. (n=8), *Aspergillus* spp. (n=4), *Acremonium* spp. (n=3), *Absidia* spp. (n=2), *Epidermophyton* spp. (n=2), *Geothrichum* spp. (n=2), *Paecilomyces* spp. (n=2), *Trichophyton* spp. (n=2), and one each of *Mucor* sp., *Chrysosporium* sp., *Cladosporium* sp., *Gliocladium* sp., *Microsporium* sp., and *Scedosporium* sp.] were obtained for culture block preparation. Identity of each organism was confirmed by culture. Each organism was grown in Sabouraud dextrose broth at 37 $^{\circ}$C for up to 10 days. Merthiolate was added to the culture at the final concentration of 0.02% (wt/vol). The organism was harvested, fixed with 10% buffered formalin, and embedded in paraffin blocks at the Department of Pathology, Ramathibodi Hospital.

Table 2.3-1. Results of the anti-CFA and anti-ELI based immunohistochemical assays, using culture blocks [paraffin-embedded blocks prepared from pure cultures of *P. insidiosum* (n=19) and true fungi (n=31); '+' indicates positive stain; '-' indicates negative stain].

Reference code	Organism identity	IHC[a]	
		Anti-CFA[b]	Anti-ELI[c]
CP01	*P. insidiosum*	+	+
CP02	*P. insidiosum*	+	+
CP03	*P. insidiosum*	+	+
CP04	*P. insidiosum*	+	+
CP05	*P. insidiosum*	+	+
CP06	*P. insidiosum*	+	+
CP07	*P. insidiosum*	+	+
CP08	*P. insidiosum*	+	+
CP09	*P. insidiosum*	+	+
CP10	*P. insidiosum*	+	+
CP11	*P. insidiosum*	+	+
CP12	*P. insidiosum*	+	+
CP13	*P. insidiosum*	+	+
CP14	*P. insidiosum*	+	+
CP15	*P. insidiosum*	+	+
CP16	*P. insidiosum*	+	+
CP17	*P. insidiosum*	+	+
CP18	*P. insidiosum*	+	+
CP19	*P. insidiosum*	+	+
CC01	*Fusarium* sp	-	-
CC02	*Fusarium* sp	-	-
CC03	*Fusarium* sp	-	-
CC04	*Fusarium* sp	-	-
CC05	*Fusarium* sp	-	-
CC06	*Fusarium* sp	-	-
CC07	*Fusarium* sp	-	-
CC08	*Fusarium* sp	-	-
CC09	*Aspergillus* sp	-	-
CC10	*Aspergillus* sp	-	-
CC11	*Aspergillus* sp	-	-
CC12	*Aspergillus* sp	-	-
CC13	*Acremonium* sp	-	-
CC14	*Acremonium* sp	-	-
CC15	*Acremonium* sp	-	-
CC16	*Absidia* sp	-	-
CC17	*Absidia* sp	-	-
CC18	*Epidermophyton* sp	-	-
CC19	*Epidermophyton* sp	-	-
CC20	*Geothrichum* sp	-	-
CC21	*Geothrichum* sp	-	-
CC22	*Paecilomyces* sp	-	-
CC23	*Paecilomyces* sp	-	-
CC24	*Trichophyton* sp	-	-
CC25	*Trichophyton* sp	-	-
CC26	*Mucor* sp	-	-
CC27	*Chrysosporium* sp	-	-
CC28	*Cladosporium* sp	-	-
CC29	*Gliocladium* sp	-	-
CC30	*Microsporium* sp	-	-
CC31	*Scedosporium* sp	-	-

[a] Immunohistochemical assay

[b] Rabbit anti-CFA (culture filtrate antigen) serum

[c] Rabbit anti-ELI (ELI025) serum

Table 2.3-2. Results of the anti-CFA and anti-ELI based immunohistochemical assays, using tissue blocks [paraffin-embedded blocks prepared from infected tissues of patients with pythiosis (n=19) and other mycoses (n=18); '+' indicates positive stain; '-' indicates negative stain].

Reference code	Organism identity	Organ/Tissue	GMS[a]	IHC[b]	
				Anti-CFA[c]	Anti-ELI[d]
TP01	*P. insidiosum*	Cornea	+	+	+
TP02	*P. insidiosum*	Artery	+	+	+
TP03	*P. insidiosum*	Artery	+	+	+
TP04	*P. insidiosum*	Artery	+	+	+
TP05	*P. insidiosum*	Artery	+	+	+
TP06	*P. insidiosum*	Artery	+	+	+
TP07	*P. insidiosum*	Artery	+	+	+
TP08	*P. insidiosum*	Brain	+	+	+
TP09	*P. insidiosum*	Cornea	+	+	+
TP10	*P. insidiosum*	Cornea	+	+	+
TP11	*P. insidiosum*	N/A[e]	+	+	+
TP12	*P. insidiosum*	N/A	+	+	+
TP13	*P. insidiosum*	N/A	+	+	+
TP14	*P. insidiosum*	N/A	+	+	+
TP15	*P. insidiosum*	N/A	+	+	+
TP16	*P. insidiosum*	N/A	+	+	+
TP17	*P. insidiosum*	N/A	+	+	+
TP18	*P. insidiosum*	N/A	+	+	+
TP19	*P. insidiosum*	N/A	+	+	+
TC01	*Aspergillus flavus*	Nasal cavity	+	-	-
TC02	*Aspergillus flavus*	Colon	+	-	-
TC03	*Aspergillus flavus*	Lung	+	-	-
TC04	*Aspergillus fumigatus*	Trachea	+	-	-
TC05	*Aspergillus fumigatus*	Air sac wall	+	-	-
TC06	*Aspergillus* sp	Sinus	+	-	-
TC07	*Aspergillus* sp	Sinus	+	-	-
TC08	*Aspergillus* sp	Nasal cavity	+	-	-
TC09	*Candida albicans*	Diaphragm	+	-	-
TC10	*Candida albicans*	Lung	+	-	-
TC11	*Candida albicans*	Lung	+	-	-
TC12	*Candida albicans*	Heart	+	-	-
TC13	*Candida* sp	N/A	+	-	-
TC14	*Candida* sp	Lip	+	-	-
TC15	*Trichosporon cutaneum*	Lung	+	-	-
TC16	Phaeomycotic fungus[f]	Skin	+	-	-
TC17	*Fusarium* sp	Cornea	+	-	-
TC18	*Fusarium* sp	Skin	+	+	-

[a] Grocott's methenamine silver stain

[b] Immunohistochemical assay

[c] Rabbit anti-CFA (culture filtrate antigen) serum

[d] Rabbit anti-ELI (ELI025) serum

[e] Data not available

[f] A pigmented fungus that causes *Phaeomycotic* cyst

A total of 37 paraffin-embedded tissue blocks, prepared from infected tissues of 19 patients with pythiosis (**Table 2.3-2**; reference codes TP01-19) and 18 patients with other fungal infections [**Table 2.3-2**; reference codes TC01-18; served as negative controls; these included *Candida albicans* (n=4), *Aspergillus* spp. (n=3), *Aspergillus flavus* (n=3), *Aspergillus fumigatus* (n=2), *Fusarium* spp. (n=2), *Candida* spp. (n=2), *Trichosporon cutaneum* (n=1), and a phaeomycotic fungus (n=1)] were obtained from Ramathibodi Hospital, Siriraj Hospital, and Chulalongkorn Hospital. The identity of each organism in the infected tissues was

confirmed by histological examination and culture identification. Each tissue or culture block was cut into 4-μm slices using a microtome (Finesse 325, Thermo Scientific, USA). Paraffin-embedded sections were placed on glass slides for downstream IHC analyses.

Grocott's methenamine silver and immunohistochemical stains: Each paraffin-embedded section was analysed with the Grocott's methenamine silver stain (GMS) as previously described (28), and examined under a light microscope (ECLIPSE Ci, Nikon, Japan). Two different IHCs for detecting *P. insidiosum* were performed, using the methods described by Keeratijarut et al (for anti-CFA based IHC) (21) and Lerksuthirat et al (for anti-ELI based IHC) (25), with some modifications. Briefly, each paraffin-embedded section was treated with xylene (to de-paraffinize) and ethanol (to replace the xylene). Slides were then washed with phosphate buffered saline (PBS, pH 7.4), and incubated in Tris-EDTA buffer (TE buffer; pH 9.0) in a water bath, at 95°C, for 40 min. To reduce nonspecific staining from endogenous peroxidase, the sections were treated with 10% H_2O_2 in PBS for 10 min, and washed with PBS. The section was incubated with 200 µl of rabbit pre-immune, anti-ELI [made available by Lerksithurat et al (25)], or anti-CFA [made available by Keeratijarut et al (21)] serum (1:16,000 in PBS) in a moisture chamber at 4 $^{\circ}$C, overnight. After washing 3 times with PBS (5 min each), the sections were incubated, at room temperature, for 30 min with 200 µl of undiluted mouse anti-rabbit IgG antibody conjugated with horseradish-peroxidase (Thermo Scientific, USA). After washing as described above, color was developed with 200 µl of 3,3'-diaminobenzidine tetrahydrochloride diluted (1:200) in DAB substrate (DAKO, USA) which was added to each section and incubated at room temperature for 5 min. The section was counterstained with hematoxylin for 15 min, and examined under a light microscope (ECLIPSE Ci, Nikon, Japan). A stain section was considered positive, if organisms were stained brown, and negative if organisms were unstained. Assay interpretation was determined by two independent examiners. Positive or negative calls were consistently interpreted by both examiners.

Statistical analysis: Detection sensitivity, detection specificity, positive predictive value (PPV), negative predictive values (NPV), and accuracy were calculated using the Microsoft Excel 2013 software (4).

2.3.3 Results

Analyses of the paraffin-embedded culture blocks and tissue blocks.

To test the sensitivity and specificity of IHCs developed to detect *P. insidiosum* in infected tissues, two different types of specimens were utilized. In one set, pure cultures of 19 independent isolates of *P insidiosum* and pure cultures of 31 independent isolates of true fungi were embedded in paraffin ('culture blocks'; see Methods; **Table 2.3-1**). Another set of paraffin-embedded block specimens was produced from tissues obtained from pythiosis patients or from patients with various mycoses ('tissue blocks'; see Methods; **Table 2.3-2**).

Before proceeding to the IHC, it was important to confirm that the paraffin-embedded culture block and tissue block sections harbored the organism in question. For the culture block sections, organisms were microscopically visible, without GMS staining (data not shown). For the tissue-block sections, GMS was used to stain the hyphal elements black for microscopic detection of infecting organisms. In all tissue block sections, including 19 *P. insidiosum* specimens and 18 specimens of true fungi, hyphal elements were visible with GMS staining (**Figure 2.3-1A & D**, and data not shown)

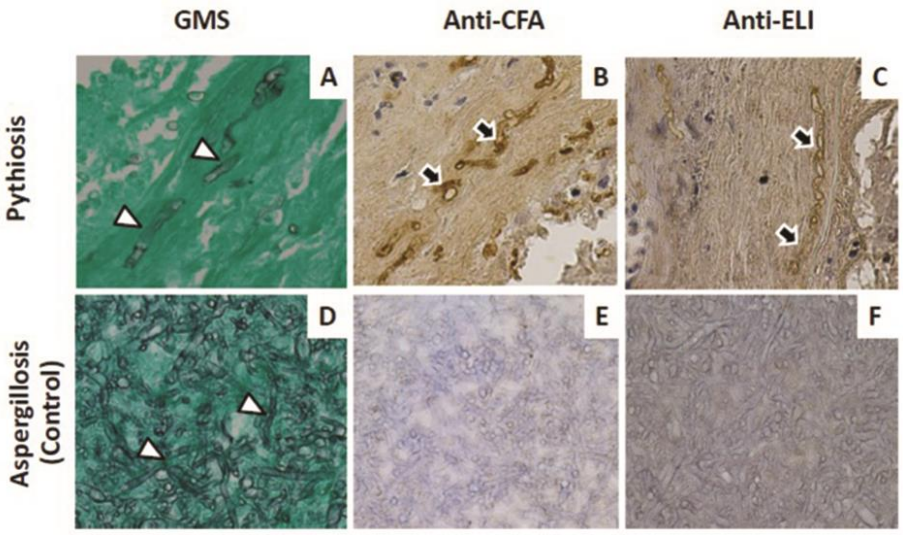

Figure 2.3-1. Immunohistochemical stains of sections from a patient with pythiosis (**A-C**; sample reference code, TP01) and aspergillosis (**D-F**; sample reference code, TC01). (**A**) and (**D**) represent Grocott's methenamine silver stain (GMS) (white arrow heads indicate organisms). (**B**) and (**E**) show immunohistochemical stain using the anti-culture filtrate antigen antibody (anti-CFA) as primary antibody (arrows indicate positive stain). (**C**) and (**F**) demonstrate immunohistochemical stain using the anti-elicitin antibody (anti-ELI) as primary antibody (black arrows indicate stained organisms).

Development of an immunohistochemical assay using anti-elicitin antibody

Early experiments were performed to establish an optimal antibody dilution for the IHC. The rabbit anti-ELI025 antibody (anti-ELI) (25), was used as primary antibody for immunohistochemical staining of *P. insidiosum* in tissue sections, and the optimal dilution was determined to be 1:16,000 (Methods; data not shown). With this dilution of anti-ELI, *P. insidiosum*'s hyphae stained brown in all of the paraffin-embedded sections tested (**Figure 2.3-1C**), whilst the IHC, using rabbit pre-immune serum, did not stain the organism (data not shown).

Comparison of anti-CFA and anti-ELI based immunohistochemical assays

The anti-CFA based IHC, using the rabbit anti-CFA antibody (21), and the anti-ELI based IHC, were evaluated for their diagnostic performance against the same set of culture block sections (**Table 2.3-1**). For both IHC assays, hyphae were clearly detected in all 19 *P. insidiosum* culture block-derived sections, and failed to stain any organisms in any of the negative-control sections (**Table 2.3-1**). Based on the results of all 50 culture blocks, both anti-CFA and anti-ELI based IHCs attained 100% responses for detection sensitivity, detection specificity, PPV, NPV, and accuracy (**Table 2.3-3**).

The anti-CFA and anti-ELI based IHCs were further evaluated, using the tissue blocks (**Table 2.3-2**). Both IHCs detected organisms in all 19 *P. insidiosum* tissue block-derived sections (**Table 2.3-2**; **Figure 2.3-1B** and **1C**). Of 18 control tissue-derived sections (**Table 2.3-2)**, 17 (reference codes TC01-17) were unstained by both IHCs (**Figure 2.3-1E** and **1F**). One section (*Fusarium*-infected tissue; reference code TC18) was stained positive by the anti-CFA based IHC, but stained negative by the anti-ELI based IHC (data not shown). Based on the results of all 37 *P. insidiosum* and control tissue blocks, the anti-ELI based IHC demonstrated 100%

detection sensitivity, detection specificity, PPV, NPV, and accuracy (**Table 2.3-3**). While the anti-CFA based IHC exhibited 100% detection sensitivity and NPV, it showed 94.4% specificity, 95.0% PPV, and 97.3% accuracy (**Table 2.3-3**).

Diagnostic performance values of both IHCs were also calculated based on the combined results of all 87 culture and tissue blocks. Detection sensitivity, detection specificity, PPV, NPV, and accuracy of the anti-ELI based IHC were all determined to be 100%, while that of the anti-CFA based IHC were 100%, 98.0%, 97.4%, 100.0%, and 98.9%, respectively (**Table 2.3-3**).

Table 2.3-3. Diagnostic performance of the anti-CFA and anti-ELI based immunohistochemical assays, evaluated against the culture (*P. insidiosum*, n=19; other fungi, n=31) and tissue (*P. insidiosum*, n=19; other fungi, n=18) blocks.

Sample type	anti-CFA based IHC[a]					anti-ELI based IHC[b]				
	Sensitivity (%)	Specificity (%)	PPV (%)[c]	NPV (%)[d]	Accuracy (%)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
Culture blocks[e]	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Tissue blocks[f]	100.0	94.4	95.0	100.0	97.3	100.0	100.0	100.0	100.0	100.0
Culture&Tissue blocks	100.0	98.0	97.4	100.0	98.9	100.0	100.0	100.0	100.0	100.0

[a] Immunohistochemical assay (IHC) using the rabbit anti-CFA (culture filtrate antigen) serum

[b] Immunohistochemical assay (IHC) using the rabbit anti-ELI (ELI025) serum

[c] Positive predictive value

[d] Negative predictive value

[e] Paraffin-embedded block prepared from pure culture of *P. insidiosum* or other fungi

[f] Paraffin-embedded block prepared from infected tissues of patients with pythiosis or other mycoses

2.3.4 Discussion

IHC is a well-known technique, routinely performed in general pathology laboratories. IHC is a useful assay for detection of *P. insidiosum* (20,21,29), especially when culture identification, serodiagnosis or molecular detection, is not available or fails to definitively diagnose the infecting organism in possible cases of pythiosis. Rabbit antiserum, raised against the *P. insidiosum* crude extract (i.e., SABH and CFA, which contain various protein species) has been used as the primary tool in development of the IHCs (20,21,29). These assays demonstrated high detection sensitivity. However, some investigators observed limited specificity of IHCs (17,21), which was conceivably due to cross reactivity, by non-specific antibodies in the rabbit antiserum, to some fungi (i.e., *Fusarium* and *Conidiobolus*) that share microscopic features with *P. insidiosum*. Improving the diagnostic performance of IHC relied on the specificity of the anti-*P. insidiosum* antibody.

Among the human pathogens, elicitins are present only in *P. insidiosum*, but not in true fungi and other microorganisms (22–27). One of the elicitins, ELI025, has been identified in *P. insidiosum*, as a highly-expressed protein on the cell surface (25–27). ELI025 has been successfully expressed and purified as a recombinant protein from the bacterium *Escherichia coli* (25). Due to the uniqueness of ELI025, rabbit antiserum (anti-ELI) raised against this protein (25) is expected to be specific to *P. insidiosum*, and therefore, a good candidate for IHC development. In addition, among different *P. insidiosum* strains isolated from humans and animals living in different geographic areas across the world, ELI025 is immunologically conserved, and can be detected by anti-ELI (30). In the present study, an anti-ELI based IHC was successfully developed, and its diagnostic performance was evaluated and compared with that of the anti-CFA based IHC (21).

Because a limited number of the paraffin-embedded tissue blocks were available (n=37; **Table 2.3-2**), some paraffin-embedded culture blocks were prepared from 19 different *P. insidiosum* strains and 31 various fungal species, including those which share hyphal morphology with *P. insidiosum* (**Table 2.3-1**). Diagnostic parameters of anti-ELI and anti-CFA based IHCs were analyzed (**Table 2.3-1** and **2**). The GMS assay (28), which stains fungal and *P. insidiosum* hyphal elements, ensured the presence of the expected organism in the sections prepared from the tissue blocks and in so doing, eliminated possible false negative results. The anti-ELI and anti-CFA based IHCs correctly detected *P. insidiosum* in all culture and tissue block-derived sections, indicating that both assays had equally 100% detection sensitivity (**Table 2.3-1, 2, and 3**). Both IHCs were negative for the control fungi in all specimens tested, except one sample, from a patient with *Fusarium* infection (**Table 2.3-2**; reference code: TC18), that was positive by the anti-CFA based IHC. This indicates that detection specificity of the anti-CFA based IHC (98%) was slightly lower than that of the anti-ELI based IHC (100%) within the sample set tested (**Table 2.3-3**). Cross reactivity between *P. insidiosum* and *Fusarium* species was also observed by Keeratijarut et al, using the same assay (21). Since only one in 10 *Fusarium* specimens tested (**Table 2.3-1** and **2;** reference codes: CC01-08 and TC17-18) was reactive against the anti-CFA antibody, it is likely that not all *Fusarium* species share antigens with *P. insidiosum*. As demonstrated here, the IHC, using anti-ELI as the refined mono-protein specific primary antibody, retained high detection sensitivity while improving detection specificity, compared to anti-CFA, the multi-protein specific primary antibodies.

In conclusion, accurate diagnosis of pythiosis is remarkably critical for proper and timely management (i.e., adequate surgical intervention), which promotes better clinical outcomes for affected patients or animals. We have successfully developed an IHC, using the anti-ELI antibodies, and improved diagnostic performance (100% sensitivity, specificity, PPV, NPV, and accuracy) for detection of *P. insidiosum* in tissues. This is an advance in the field of *P. insidiosum* studies, where basic biological knowledge and genetic engineering technology, have facilitated the successful development of a better diagnostic assay.

2.3.5 References

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2.4 Protein A/G-based immunochromatographic test for serodiagnosis of pythiosis

2.4.1 Introduction

Pythiosis is a life-threatening infectious disease caused by the fungus-like, aquatic, oomycete organism *Pythium insidiosum*.[1-5] Most cases of pythiosis have been reported in humans, horses, dogs, cats, and cattle, but some other domestic and wild animals are also infected.[2,4,5] Tropical wetlands are the natural habitat of *P. insidiosum*, and the disease is likely to be acquired through the ingestion of contaminated water or direct contact with the pathogen to host surfaces.[6,7] Pythiosis has been found in Asian countries, i.e., Thailand, Malaysia, and India.[4,8,9] However, the disease has been reported in the Americas (i.e., U.S.A., Costa Rica, Brazil),[10-12] Africa,[13] and parts of Australia and New Zealand.[14-16] Various forms of pythiosis have been observed, depending on the site at which the infection initiates, i.e., artery, eye, skin, and gastrointestinal tract. Cutaneous/subcutaneous infection is the most common form of pythiosis in horses, whereas in dogs, gastrointestinal tract infection is more prevalent. In humans, *P. insidiosum* infections of arteries (vascular pythiosis) and eyes (ocular pythiosis) have been frequently reported.

Pythiosis has a high rate of mortality and morbidity.[2-5] Use of antifungal drugs are ineffective for the treatment of pythiosis. Extensive surgical removal of infected tissues is the main treatment option for cure. Prompt and effective treatment is required to promote better outcome for the affected individuals, and this could be achieved by early and accurate diagnosis of pythiosis. Several diagnostic methods are available for pythiosis.[2,4,5] Culture identification and PCR-based diagnostic assays are used for the direct detection of *P. insidiosum* in clinical samples.[8,17-22] However, these methods are time-consuming and require experienced personnel. In addition, culture identification often fails to isolate the organism from the infected tissue sample. Alternatively, serodiagnostic assays, including immunodiffusion (ID),[23,24] enzyme-linked immunosorbent assay (ELISA),[25-29] hemagglutination (HA),[30] and immunochromatographic test (ICT),[31] have been developed for detection of anti-*P. insidiosum* antibodies in serum samples. Among these assays, ELISA and ICT showed most favorable diagnostic performance for pythiosis.[28]

As alluded to earlier, pythiosis has been increasingly diagnosed in mammals, including humans and various domestic and wild animals worldwide.[2-5] There is a need for a serological test that could facilitate the diagnosis of *P. insidiosum* infections in these mammalian subjects. While ID is relatively slow and insensitive, HA has a poor diagnostic performance, and ELISA requires a multi-step procedure and expensive equipment, ICT appears to be a rapid, user-friendly, and efficient test format for serodiagnosis of pythiosis.[23-28,30,31] ICT has been developed to detect anti-*P. insidiosum* antibodies in serum samples particularly from human patients, and not that from other animals.[31] This limitation is, however, due to different reagents (i.e., host-specific anti-IgG antibodies) are needed to perform the test against sera obtained from different hosts. By using the bacterial protein A/G that binds various mammalian IgGs,[32,33] the present study aims to develop an ICT, using the bacterial protein A/G, to detect anti-*P. insidiosum* IgGs in humans and animals, and compare its performance with the established ELISA.[26,28,34]

2.4.2 Methods

Serum samples: A total of 85 serum samples from 28 human patients (26 vascular, 1 ocular, and 1 cutaneous pythiosis), 24 dogs, 12 horses, 12 rabbits, and 9 cattle with pythiosis were recruited for ICT and ELISA analyses.[26,28,31,34] Diagnosis of pythiosis was based on: (i) culture identification of *P. insidiosum* from

clinical specimens;[17] (ii) PCR-based assay or sequence homology analysis of *P. insidiosum* rDNA;[8,18,20] or (iii) detection of anti–*P. insidiosum* antibodies in serum samples by established serodiagnostic tests.[23-28,30,31,34] To serve as the control, a total of 143 serum samples were recruited from 80 human subjects (healthy blood donors [n=62] and patients with thalassemia [n=10], aspergillosis [n=3], zygomycosis [n=2], candidiasis [n=1], cryptococcosis [n=1], and histoplasmosis [n=1]), 31 dogs (healthy dogs [n=9], dogs with *Lagenidium giganteum* forma *caninum* infection [n=8], *Paralagenidium karlingii* infection [n=6], zygomycosis [n=3], aspergillosis [n=1], blastomycosis [n=1], cryptococcosis [n=1], protothecosis [n=1], and sporotrichosis [n=1]), 12 healthy cattle, 10 healthy horses, and 10 healthy rabbits. All sera were kept frozen until use.

Antigen preparation: The *P. insidiosum* strain Pi-S, isolated from a Thai patient with pythiosis, was maintained on Sabouraud dextrose (SD) agar, and subcultured (at 37 °C) once a month until use. The antigen was prepared, using the protocol described by Krajaejun et al.[35] Briefly, several small pieces of SD agar containing growing *P. insidiosum* mycelium were cultured (at 37 °C for 10 days) with shaking (150 rpm) in a flask containing 200 ml of SD broth. The organism was killed by adding Thimerosal [final concentration: 0.02% (wt/vol)], and separated from cultured SD broth by filtration through a 0.22-μm pore size membrane (Durapore). To prepare the culture filtrate antigen (CFA), after adding protease inhibitors [PMSF (0.1 mg/ml) and EDTA (0.3 mg/ml)], the cell-free SD broth was concentrated ~80 fold using the Amicon 8400 apparatus and an Amicon Ultra-15 centrifugal filter (Millipore). Protein concentration of CFA was estimated by spectrophotometry. The CFA was stored at -20 ºC until use.

Protein A/G-based immunochromatographic test: The protein A/G based ICT was produced at the Chulabhorn Research Institute and involved the following step:

(I) Preparation of protein A/G colloidal gold conjugate: Protein A/G (Prospec, Ness-Ziona, Israel) was coupled to a colloidal gold particle by pI-dependent passive adsorption. The 40-nm colloidal gold solution (Arista, Allentown, PA) was adjusted to pH 7.2 with 0.2 M Na_2CO_3 under gentle stirring. The gold suspension was divided into aliquots of 0.5 ml in 1.5 ml microcentrifuge tubes to which 30 μl of protein A/G (0.1 mg/mL) was added with gentle vortexing. The mixture was allowed to conjugate at room temperature for 30 min. The residual surface of the gold particle was blocked with 125 μL of 5% (wt/vol) casein dissolved in 5 mM sodium phosphate buffer (SPB) pH 7.4 for 15 min. The conjugation mixture was centrifuged at 6,000 x g at room temperature for 15 min and the supernatant was discarded. The pellet was washed with 0.5% (wt/vol) casein and the suspended conjugated particle was centrifuged again under identical settings. After removing the supernatant, the pellet was re-suspended in a solution of 0.5% (wt/vol) casein containing 20% (wt/vol) sucrose in 5 mM SPB pH 7.4 to 2.5% of the original volume of colloidal gold suspension. The protein A/G colloidal gold conjugate (2.5 μL) was transferred to a piece of 2.5x4.0 mm glass fiber filter GF33; (Whatman Schleicher & Schuell, Dassel, Germany). The impregnated glass fiber was dried in a dehumidifier cabinet for 2 hours and was used to construct the ICT.

(II) Immobilization of antigen and antibody onto a nitrocellulose membrane: Immobilization of proteins on nitrocellulose membrane (NCmb) (AE99; Whatman Schleicher & Schuell, Dassel, Germany) was performed by passive physical adsorption in line pattern. A BioDot ZX1000™ dispensing platform (BioDot, Irvine, CA) was used for this purpose. The transfer rate of the solution was set at 1 μl/cm. A 1.25x20 cm nitrocellulose membrane was lined along its length with 1 mg/mL CFA to form a test line and with 0.2 mg/mL normal rabbit IgG in 50 mM ammonium acetate buffer pH 4.5 to form a control line. The protein immobilized

membrane was dried and blocked with 1% (wt/vol) BSA, 0.1% (wt/vol) trehalose in 10 mM SPB pH 7.4 and dried again in a dehumidifier cabinet.

(III) Preparation of the sample pad: The sample pad used in the ICT strip was made of paper (#903 Whatman Schleicher & Schuell, Dassel, Germany) previously immerged in 2% (wt/vol) Triton X-100, 0.05% (wt/vol) polyvinylpyrrolidone (PVP) in 50 mM Tris-HCl, pH 7.4 and dried in a dehumidifier cabinet.

(IV) Composition and construction of the ICT strip: The ICT strip system was assembled by utilizing five major components: the sample pad, the glass fiber impregnated with colloidal gold conjugate, the protein immobilized NC membrane, a wicking pad (3MM chromatography paper, Whatman, Maidstone, England) and the plastic backing. The first 4 components were assembled with 1-2 mm overlap on the plastic backing support (G&L; San Jose, CA). The assembled card was then cut into 2.5 mm wide strips with a strip cutting machine (CM 4000 R; BioDot, Irvine, CA) (**Figure 2.4-1**).

(V) Detection of anti-*P. insidiosum* antibodies in human and animal sera: The ICT assay was carried out in a 96-well microtiter plate or a microtube. Human sera were diluted 1:10,000 (in 0.15 M PBS pH 7.4) and tested with ICT in Center #1 (Ramathibodi Hospital, Thailand), while animal sera were diluted 1:5,000 (in 0.15 M PBS pH 7.4) and tested with ICT in Center #2 (for samples from dogs; Louisiana State University, USA) and Center #3 (for samples from horses, dogs, cattle, and rabbits; Universidade Federal de Santa Maria, Brazil). The protein A/G-based ICT strip was dipped into a well containing 0.1 mL of each diluted serum sample in duplicate. The serum sample moved through the sample pad and the conjugate pad by capillary force. The mixture moved along the membrane immobilized with CFA acting as the test line. If anti-*P. insidiosum* antibody is present in the serum sample, the result is the formation of colored bands of colloidal gold conjugate at the test line and also the control line. On the other hand, a negative sample gives only one band at the control line. The developed signal of each ICT was read visually at 30 min. Detection sensitivity, detection specificity, and assay accuracy were calculated using the Microsoft EXCEL2013 program.

Enzyme-linked immunosorbent assay: ELISA for detection of anti-*P. insidiosum* antibodies in serum samples was carried out in three centers: (i) Department of Pathology, Faculty of Medicine, Ramathibodi Hospital, Mahidol University, Bangkok, Thailand (Center #1), using the ELISA protocol of Chareonsirisuthigul et al[28] for testing human sera; (ii) Department of Veterinary Clinical Sciences, Louisiana State University, Baton Rouge, LA, USA (Center #2), using the ELISA protocol of Grooters et al[26] for testing dog sera; and (iii) Depto Microbiologia, Universidade Federal de Santa Maria, Santa Maria, RS, Brazil (Center #3), using the ELISA protocol of Santurio et al[34] for testing sera from horses, dogs, cattle, and rabbits. The *P. insidiosum* antigen used for the ELISA performed in Center #1 was CFA (exoantigen),[28] while that used for the ELISA performed in Center #2 and Center #3 was soluble hyphal antigen.[26,34] The ELISA cut-off value was calculated based on the mean optical density (OD) of the control sera plus three SDs (Center #1 and #3), or the mean percent positivity of all control sample ODs (in relation to the strong positive control serum OD) plus three SDs (Center #2). Any samples with ELISA values above the cut-off were determined to be positive, while those below the cut-off were determined to be negative. Detection sensitivity, detection specificity, assay accuracy, and ELISA cut-off values were calculated using the Microsoft EXCEL2013 program.

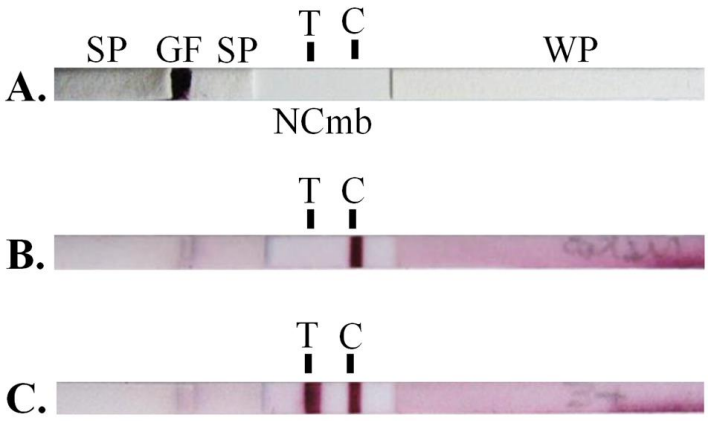

Figure 2.4-1. **Schematic diagrams of the proteins A/G based immunochromatography (ICT) for detection of anti-*Pythium insidiosum* IgGs.** Panel **A** (actual ICT strip) shows an untested ICT (either test or control line is not generated). Panel B depicts a negative result (only the control line is visible), whereas panel **C** exhibits a positive result (both test and control lines are visible). (Abbreviations: SP, sample pad; GF, glass fiber; WP, wicking pad; NCmb, nitrocellulose membrane; T, test line; and C, control line)

2.4.3 Results

Development of a protein A/G-based ICT: An assembled protein A/G-based ICT is shown in **Figure 2.4-1**. CFA (crude protein extract from *P. insidiosum* strain Pi-S) and the commercially-available normal rabbit IgGs were separately streaked as a straight line on a nitrocellulose membrane, and served as 'test' and 'control' lines, respectively. Various IgG species in a human or animal serum samples were absorbed and migrated along the sample pad, and then the glass fiber, where complexes of IgGs and protein A/G conjugated with colloidal gold were formed. The IgG-protein A/G-colloidal gold complexes moved through the nitrocellulose membrane. The complexes containing anti-*P. insidiosum* IgGs captured the CFA blotted at the test line, and developed a purple signal of accumulated colloidal golds. The complexes lacking anti-*P. insidiosum* IgGs passed through the test line, without developing any signal. The normal rabbit IgGs, blotted at the control line, bound the protein A/G in the remaining complexes, and developed a purple signal.

After distribution of the ICT from Center #1 (Thailand) to Center #2 (U.S.A.) and Center #3 (Brazil), the assay can still function properly. After two years of storage at room temperature, the ICT can still effectively detect the anti-*P. insidiosum* IgGs in serum samples.

Performance comparison of ICT and ELISA: The established ELISA[26,28,34] and the protein A/G-based ICT had been independently performed to determine anti-*P. insidiosum* antibodies in sera from 108 humans, 55 dogs, 22 horses, 21 cattle, and 22 rabbits, with (n=85) or without (n=143; served as control) pythiosis, in the three centers: Center #1 (Department of Pathology, Ramathibodi Hospital, Mahidol University, Bangkok, Thailand), Center #2 (Department of Veterinary Clinical Sciences, Louisiana State University, Baton Rouge, LA, USA), and Center #3 (Depto Microbiologia, Universidade Federal de Santa Maria, Santa Maria, RS, Brazil) (**Table 2.4-1**). The test results (i.e., ELISA value ranges, means, and cutoffs) and diagnostic performances (i.e., sensitivities, specificities, and accuracies) of ELISA and the protein A/G-based ICT, according to host types (humans, dogs, horses, cattle, and rabbits), disease states (pythiosis or control), and assay-performing centers, are summarized in **Table 2.4-1**.

All control serum samples (n=143) were tested negative by both the ELISA and protein A/G-based ICT. Among all 85 pythiosis sera, 77 samples were consistently tested positive by both serological assays. ELISA and ICT failed to detect anti-*P. insidiosum* antibodies in the same serum from a patient with ocular pythiosis (ELISA value: 1.91; ELISA cutoff: 4.45). Two patients with vascular pythiosis (ELISA values: 11.60 and 7.08), one patient with cutaneous pythiosis (ELISA value: 7.59), three horses with pythiosis (ELISA values: 0.26, 0.25, and 0.22; ELISA cutoff: 0.21), and a Center #2 dog with pythiosis (ELISA value: 16.73; ELISA cutoff: 10.02) were tested positive by ELISA, but negative by ICT. One pythiosis serum from a human subject was weakly positive by ICT and ELISA (ELISA values: 6.28). Based on the results of all pythiosis and control sera, regardless of host types and assay-performing centers, ELISA showed 98.8% detection sensitivity, 100% detection specificity, and 99.6% accuracy, while ICT showed 90.6% detection sensitivity, 100% detection specificity, and 96.5% accuracy.

Table 2.4-1. Diagnostic performances of ICT and ELISA tested against serum samples from humans (n=108) or animals (n=120), with and without pythiosis, in Center #1 (located in Thailand), Center #2 (U.S.A.), and Center #3 (Brazil).

Center	Host (Total sera)	Range of ELISA values (Mean)		ELISA cutoff value	ELISA			ICT		
		Pythiosis	Control		TP [a] / PC [b] (Sensitivity, %)	TN [c] / CC [d] (Specificity, %)	Accuracy (%) [e]	TP / PC (Sensitivity, %)	TN / CC (Specificity, %)	Accuracy (%)
#1	Humans (108)	20.25 - 1.91 (13.47)	4.45 - 0.33 (1.55)	4.45	27/28 (96.4)	80/80 (100.0)	99.1	24/28 (85.7)	80/80 (100.0)	96.3
#2	Dogs (44)	105.70 - 16.73 (62.38)	9.11 - 2.68 (5.01)	10.02	18/18 (100.0)	26/26 (100.0)	100.0	17/18 (94.4)	26/26 (100.0)	97.7
#3	Dogs (11)	1.37 - 1.16 (1.25)	0.75 - 0.30 (0.47)	1.00	6/6 (100.0)	5/5 (100.0)	100.0	6/6 (100.0)	5/5 (100.0)	100.0
	Horses (22)	1.11 - 0.22 (0.80)	0.21 - 0.12 (0.17)	0.21	12/12 (100.0)	10/10 (100.0)	100.0	9/12 (75.0)	10/10 (100.0)	86.4
	Cattle (21)	0.37 - 0.17 (0.23)	0.16 - 0.09 (0.13)	0.16	9/9 (100.0)	12/12 (100.0)	100.0	9/9 (100.0)	12/12 (100.0)	100.0
	Rabbits (22)	0.33 - 0.19 (0.27)	0.15 - 0.09 (0.12)	0.18	12/12 (100.0)	10/10 (100.0)	100.0	12/12 (100.0)	10/10 (100.0)	100.0
#1 - 3	Overall (228)	N/A [f]	N/A	N/A	84/85 (98.8)	143/143 (100.0)	99.6	77/85 (90.6)	143/143 (100.0)	96.5

Footnote:

[a] Number of cases with true positive (TP) results

[b] Pythiosis cases

[c] Number of cases with true negative (TN) results

[d] Control cases

[e] Accuracy (%), [(all cases with true positive and true negative results) / (all pythiosis and control cases)] x 100

[f] N/A, not applicable

2.4.4 Discussion

The protein A/G-based ICT was successfully developed for the detection of specific anti-*P. insidiosum* IgGs in serum samples from humans and animals with pythiosis, which is a striking advantage over the previously-reported ICT that can detect only the IgGs from human subjects.[31] The diagnostic performance of ICT was compared to that of ELISA, a highly-efficient assay established for the serodiagnosis of pythiosis in humans or animals.[26,28,34] Interpretation of ICT results depends on the presence or absence of the test line (which is subjectively read by the naked eye; **Figure 2.4-1**), while interpretation of ELISA results depends on an OD value above or below the cutoff (which was objectively quantitated by an ELISA plate reader). ICT and ELISA did not detect the anti-*P. insidiosum* IgG antibodies in the control sera from healthy individuals, as well as those from humans and animals with infections caused by other pathogens, including those share microscopic morphologies with *P. insidiosum* (i.e., *Lagenidium*, *Paralagenidium*, *Aspergillus*, and Zygomycetes) (**Table 2.4-1**). This finding indicates that both ELISA and ICT had no cross-reactivity with other pathogens, and thus, provides equivalently-high detection specificity (100%).

Regardless of the sources of the sera tested, the overall detection sensitivity of ICT was considered high (~91%), although slightly lower than that of ELISA (~99%; **Table 2.4-1**). This was due to some serum samples from several proven cases of human (n=4), equine (n=3), and canine (n=1) pythiosis, being read

negatively by ICT (i.e., no visible test line), but marginally positive by ELISA (i.e., ELISA values that were slightly above the cutoff; **Table 2.4-1**). The subjective nature of result interpretation could explain the limited detection sensitivity of ICT, especially when fewer anti-*P. insidiosum* antibodies were present in the serum sample, as indicated by a low ELISA value. The possibility of the presence of anti-protein A/G antibodies in the serum, that inhibits the formation of IgG-protein A/G-colloidal gold complexes, and leads to an absence of the test line, is unlikely. This can be explained by the fact that the ICT control line, generated by complex formation of the normal rabbit IgGs and the protein A/G conjugated with colloidal gold (**Figure 2.4-1**), was strongly developed in all serum samples tested, indicating that there was no anti-bacterial protein A/G antibodies in the samples. The reason for the false negative results was most likely due to the lower detection sensitivity of the ICT, as compared to that of ELISA. Poor host antibody responses can be observed in localized infections of the eye,[30,31] and could explain the failure of ICT and ELISA to detect anti-*P. insidiosum* antibodies in the serum from patient with ocular pythiosis. Therefore, the use of neither ICT nor ELISA is recommended for making a diagnosis of ocular pythiosis.

ELISA and ICT showed high accuracy (99.6% and 96.5%, respectively; **Table 2.4-1**), indicating that both assays reliably reported the true positive and true negative results. Here, we showed that ELISA is highly sensitive for the diagnosis of pythiosis, which was consistent with the reports of other investigators.[25-28,34] However, ELISA has a long turnaround time (> 3 hr), and to perform this multi-step assay, it requires a specific secondary antibody (for each host type), experienced personnel, and special equipment. Such limitations of ELISA could be addressed by the successful development of the protein A/G-based ICT, which appears to be rapid (shorter turnaround time: ~30 min), highly-sensitive (91%), and easy-to-use assay that can facilitate serodiagnosis of pythoisis, especially in non-reference laboratories.

In conclusion, an ICT has been successfully developed for the serodiagnosis of humans and animals with pythiosis. ICT has a high detection sensitivity (91%), detection specificity (100%), and accuracy (97%). Yet, it was designed and manufactured to be a rapid, user-friendly, and efficient test. The current ICT has a long shelf storage life (at least two years), and it can be distributed worldwide, without effecting its performance. ICT could facilitate the diagnosis of pythiosis in most cases. However, if ICT is read negative in a suspected case of pythiosis, further analysis using a more sensitive assay (i.e., ELISA) is recommended.

2.4.5 References

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3. Outputs of the project

3.1 Publications (*corresponding author)

(1) Lerksuthirat T, Lohnoo T, Inkomlue R, Rujirawat T, Yingyong W, Khositnithikul R, Phaonakrop N, Roytrakul S, Sullivan TD, **Krajaejun T***. The elicitin-like glycoprotein, ELI025, is secreted by the pathogenic oomycete *Pythium insidiosum* and evades host antibody responses. PLoS One. 2015; 10(3):e0118547.

(2) Lerksuthirat T, Lohnoo T, Rujirawat T, Yingyong W, Jongruja N, **Krajaejun T***. Geographic variation in the elicitin-like glycoprotein, ELI025, of *Pythium insidiosum* isolated from human and animal subjects. Infect Genet Evol. 2015; 35:127-33.

(3) Inkomlue R, Larbcharoensub N, Karnsombut P, Lerksuthirat T, Aroonroch R, Lohnoo T, Yingyong W, Santanirand P, Sansopha L, **Krajaejun T***. Development of an Anti-Elicitin Antibody-Based Immunohistochemical Assay for Diagnosis of Pythiosis. J Clin Microbiol. 2016; 54(1):43-8.

(4) Intaramat A, Sornprachum T, Chantrathonkul B, Chaisuriya P, Lohnoo T, Yingyong W, Jongruja N, Kumsang Y, Sandee A, Chaiprasert A, Banyong R, Santurio JM, Grooters AM, Ratanabanangkoon K*, **Krajaejun T***. Protein A/G-based immunochromatographic test for serodiagnosis of pythiosis in human and animal subjects from Asia and Americas. Med Mycol. 2016; 54(6):641-7.

3.2 Patent

Inkomlue R, Larbcharoensub N, Karnsombut P, Lerksuthirat T, Aroonroch R, Lohnoo T, Yingyong W, Santanirand P, Sansopha L, **Krajaejun T**; Mahidol University, assignee. [Detection of *Pythium insidiosum* by Immunohistochemical Assay]. Thailand patent application no. 1501006642. 2015 Oct 30. Thai.

3.3 Conference presentations

(1) Oral presentation (Topic: Development of an Anti-Elicitin Antibody-based Immunohistochemical Assay for Diagnosis of Pythiosis)
Meeting: the 2015 New Zealand Microbiology Society (NZMS) conference (Rotorua, New Zealand, 2-5 November 2015)

(2) Oral presentation (Topic: Elicitin-like Glycoprotein, ELI025, is Secreted by the Pathogenic Oomycete *Pythium insidiosum* and Evades Host Antibody Responses)
Meeting: The 2014 Oomycete Molecular Genetics Network (OMGN) meeting (Norwich, United Kingdom, 2-4 July 2014)

3.4 Students and young researchers

(1) Graduated PhD student: Ms. Tassanee Lerksuthirat (PhD in Molecular Medicine)

(2) Graduated MSc student: Ms. Ruchuros Inkomlue (MSc in Clinical Pathology)

3.5 Established collaborators

(1)	Dr. Rangsima Aroonroch	Faculty of Medicine, Ramathibodi Hospital, Mahidol University
(2)	Dr. Noppadol Larbcharoensub	Faculty of Medicine, Ramathibodi Hospital, Mahidol University
(3)	Ms. Patcharee Karnsombut	Faculty of Medicine, Ramathibodi Hospital, Mahidol University
(4)	Dr. Pitak Santanirand	Faculty of Medicine, Ramathibodi Hospital, Mahidol University
(5)	Dr. Angkana Chaiprasert	Faculty of Medicine, Siriraj Hospital, Mahidol University
(6)	Ms. Ramrada Banyong	Faculty of Medicine, Siriraj Hospital, Mahidol University
(7)	Dr. Thareerat Kalambaheti	Faculty of Tropical Medicine, Mahidol University
(8)	Dr. Lalana Sansopha	Faculty of Medicine, Chulalongkorn University
(9)	Dr. Kavi Ratanabanangkoon	Laboratory of Immunology, Chulabhorn Research Institute
(10)	Mr. Akarin Intaramatq	Laboratory of Immunology, Chulabhorn Research Institute
(11)	Dr. Sittiruk Roytrakul	Proteomics Research Laboratory, Genome Institute, NSTDA
(12)	Ms. Narumon Phaonakrop	Proteomics Research Laboratory, Genome Institute, NSTDA
(13)	Thomas D. Sullivan	University of Wisconsin-Madison, USA
(14)	Janio M. Santurio	Universidade Federal de Santa Maria, Brazil
(15)	Amy M. Grooters	Louisiana State University, USA

4. Appendix

4.1 Patent application

4.2 Reprints

สำเนา

คำขอรับสิทธิบัตร/อนุสิทธิบัตร

☑ การประดิษฐ์
☐ การออกแบบผลิตภัณฑ์
☐ อนุสิทธิบัตร

ข้าพเจ้าผู้ลงลายมือชื่อในคำขอรับสิทธิบัตร/อนุสิทธิบัตรนี้ ขอรับสิทธิบัตร/อนุสิทธิบัตร ตามพระราชบัญญัติสิทธิบัตร พ.ศ 2522 แก้ไขเพิ่มเติมโดยพระราชบัญญัติสิทธิบัตร (ฉบับที่ 2) พ.ศ 2535 และ พระราชบัญญัติสิทธิบัตร (ฉบับที่ 3) พ.ศ 2542

สำหรับเจ้าหน้าที่

วันรับคำขอ 30 มี.ค. 2558	เลขที่คำขอ 1501006642
วันยื่นคำขอ 30 มี.ค. 2558	

สัญลักษณ์จำแนกการประดิษฐ์ระหว่างประเทศ

ใช้กับแบบผลิตภัณฑ์

ประเภทผลิตภัณฑ์

วันประกาศโฆษณา	เลขที่ประกาศโฆษณา
วันออกสิทธิบัตร/อนุสิทธิบัตร	เลขที่สิทธิบัตร/อนุสิทธิบัตร

ลายมือชื่อเจ้าหน้าที่

1.ชื่อที่แสดงถึงการประดิษฐ์/การออกแบบผลิตภัณฑ์

ชุดตรวจเชื้อก่อโรคพิธิโอซิส (Pythiosis)

2.คำขอรับสิทธิบัตรการออกแบบผลิตภัณฑ์นี้เป็นคำขอสำหรับแบบผลิตภัณฑ์อย่างเดียวกันและเป็นคำขอลำดับที่

ในจำนวน คำขอ ที่ยื่นในคราวเดียวกัน

3.ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตร และที่อยู่ (เลขที่ ถนน ประเทศ)

มหาวิทยาลัยมหิดล
เลขที่ 999 ถนนพุทธมณฑลสาย 4 ต.ศาลายา อ.พุทธมณฑล
จ.นครปฐม 73170

3.1 สัญชาติ ไทย
3.2 โทรศัพท์
3.3 โทรสาร
3.4 อีเมล์

4.สิทธิในการขอรับสิทธิบัตร/อนุสิทธิบัตร

☐ ผู้ประดิษฐ์/ผู้ออกแบบ ☑ ผู้รับโอน ☐ ผู้ขอรับสิทธิโดยเหตุอื่น

5.ตัวแทน(ถ้ามี)/ที่อยู่ (เลขที่ ถนน จังหวัด รหัสไปรษณีย์)

ดูที่หน้า 3

5.1 ตัวแทนเลขที่
5.2 โทรศัพท์ 02 644 7800 ต่อ 301
5.3 โทรสาร 02 644 8980
5.4 อีเมล์ Ploypann.chi@mahidol.ac.th

6.ผู้ประดิษฐ์/ผู้ออกแบบผลิตภัณฑ์ และที่อยู่ (เลขที่ ถนน ประเทศ)

ดูที่หน้า 3

7. คำขอรับสิทธิบัตร/อนุสิทธิบัตรนี้แยกจากหรือเกี่ยวข้องกับคำขอเดิม

ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตร ขอให้ถือว่าได้ยื่นคำขอรับสิทธิบัตร/อนุสิทธิบัตรนี้ ในวันเดียวกับคำขอรับสิทธิบัตร เลขที่ วันยื่น เพราะคำขอรับสิทธิบัตร/อนุสิทธิบัตรนี้แยกจากหรือเกี่ยวข้องกับคำขอเดิมเพราะ

☐ คำขอเดิมมีการประดิษฐ์หลายอย่าง ☐ ถูกคัดค้านเนื่องจากผู้ขอไม่มีสิทธิ ☐ ขอเปลี่ยนแปลงประเภทของสิทธิ

หมายเหตุ ในกรณีที่ไม่อาจระบุรายละเอียดได้ครบถ้วน ให้จัดทำเป็นเอกสารแนบท้ายแบบพิมพ์นี้โดยระบุหมายเลขกำกับข้อและหัวข้อที่แสดงรายละเอียดเพิ่มเติมดังกล่าวด้วย

8.การยื่นคำขอนอกราชอาณาจักร

วันยื่นคำขอ	เลขที่คำขอ	ประเทศ	สัญลักษณ์จำแนกการประดิษฐ์ระหว่างประเทศ	สถานะคำขอ
8.1				
8.2				
8.3				

8.4 ☐ ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตรขอสิทธิให้ถือว่าได้ยื่นคำขอนี้ในวันที่ได้ยื่นคำขอรับสิทธิบัตร/อนุสิทธิบัตรในต่างประเทศเป็นครั้งแรกโดย
☐ ได้ยื่นเอกสารหลักฐานพร้อมคำขอนี้ ☐ ขอยื่นเอกสารหลักฐานหลังจากวันยื่นคำขอนี้

9.การแสดงการประดิษฐ์ หรือการออกแบบผลิตภัณฑ์ ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตรได้แสดงการประดิษฐ์ที่หน่วยงานของรัฐเป็นผู้จัด
วันแสดง วันเปิดงานแสดง ผู้จัด

10.การประดิษฐ์เกี่ยวกับจุลชีพ

10.1 เลขทะเบียนฝากเก็บ	10.2 วันที่ฝากเก็บ	10.3 สถาบันฝากเก็บ/ประเทศ

11.ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตร ขอยื่นเอกสารภาษาต่างประเทศก่อนในวันยื่นคำขอนี้ และจะจัดยื่นคำขอรับสิทธิบัตร/อนุสิทธิบัตรนี้ที่จัดทำเป็นภาษาไทยภายใน 90 วัน นับจากวันยื่นคำขอนี้ โดยขอยื่นเป็นภาษา
☐ อังกฤษ ☐ ฝรั่งเศส ☐ เยอรมัน ☐ ญี่ปุ่น ☐ อื่นๆ

12.ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตร ขอให้อธิบดีประกาศโฆษณาคำขอรับสิทธิบัตร หรือรับจดทะเบียน และประกาศโฆษณาอนุสิทธิบัตรนี้ หลังจากวันที่ เดือน พ.ศ
☐ ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตรขอให้ใช้รูปเขียนหมายเลข ในการประกาศโฆษณา

13.คำขอรับสิทธิบัตร/อนุสิทธิบัตรนี้ประกอบด้วย
ก. แบบพิมพ์คำขอ 3 หน้า
ข. รายละเอียดการประดิษฐ์ 5 หน้า
ค. ข้อถือสิทธิ 1 หน้า
ง. รูปเขียน 3 รูป 3 หน้า
จ. ภาพแสดงแบบผลิตภัณฑ์
☐ รูปเขียน รูป หน้า
☐ ภาพถ่าย รูป หน้า
ฉ. บทสรุปการประดิษฐ์ 1 หน้า

14.เอกสารประกอบคำขอ
☐ เอกสารแสดงสิทธิในการขอรับสิทธิบัตร/อนุสิทธิบัตร
☐ หนังสือรับรองการแสดงการประดิษฐ์/การออกแบบผลิตภัณฑ์
☑ หนังสือมอบอำนาจ
☐ เอกสารรายละเอียดเกี่ยวกับจุลชีพ
☐ เอกสารการขอนับวันยื่นคำขอในต่างประเทศเป็นวันยื่นคำขอในประเทศไทย
☐ เอกสารขอเปลี่ยนแปลงประเภทของสิทธิ
☐ เอกสารอื่น ๆ

15. ข้าพเจ้าขอรับรองว่า
☑ การประดิษฐ์นี้ไม่เคยยื่นขอรับสิทธิบัตร/ อนุสิทธิบัตรมาก่อน
☐ การประดิษฐ์นี้ได้พัฒนาปรับปรุงมาจาก.....

16.ลายมือชื่อ (☐ ผู้ขอรับสิทธิบัตร / อนุสิทธิบัตร; ☑ ตัวแทน)

(นางสาวฉวีวรรณ จินดาธรรม)

หมายเหตุ บุคคลใดยื่นขอรับสิทธิบัตรการประดิษฐ์หรือการออกแบบผลิตภัณฑ์ หรืออนุสิทธิบัตร โดยการแสดงข้อความอันเป็นเท็จแก่พนักงานเจ้าหน้าที่ เพื่อให้ได้ไปซึ่งสิทธิบัตรหรืออนุสิทธิบัตร ต้องระวางโทษจำคุกไม่เกินหกเดือน หรือปรับไม่เกินห้าพันบาท หรือทั้งจำทั้งปรับ

5.ตัวแทน (ถ้ามี)/ที่อยู่ (เลขที่ ถนน จังหวัด รหัสไปรษณีย์)

5.1 นางสาวพลอยพรรณ จิตรแจ้ง ตัวแทนเลขที่ 2254 และ/หรือ

5.2 นางสาวฉวีวรรณ จินดาธรรม ตัวแทนเลขที่ 2414

อยู่ที่ สถาบันวิวัฒน์เทคโนโลยีและนวัตกรรมแห่งมหาวิทยาลัยมหิดล ที่อยู่ 999 ถนนพุทธมณฑล สาย 4 ตำบลศาลายา อำเภอพุทธมณฑล จังหวัดนครปฐม 73170 ประเทศไทย

6.ผู้ประดิษฐ์/ผู้ออกแบบผลิตภัณฑ์ และที่อยู่ (เลขที่ ถนน ประเทศ)

6.1 นายแพทย์ธีรพงษ์ กระแจะจันทร์	อยู่ที่ ภาควิชาพยาธิวิทยา คณะแพทยศาสตรรามาธิบดี มหาวิทยาลัยมหิดล เลขที่ 270 ถนนพระรามหก เขตราชเทวี กทม. 10400
6.2 ดร.พิทักษ์ สันตนิรันดร์	อยู่ที่ ภาควิชาพยาธิวิทยา คณะแพทยศาสตรรามาธิบดี มหาวิทยาลัยมหิดล เลขที่ 270 ถนนพระรามหก เขตราชเทวี กทม. 10400
6.3 นายแพทย์นภดล ลาภเจริญทรัพย์	อยู่ที่ ภาควิชาพยาธิวิทยา คณะแพทยศาสตรรามาธิบดี มหาวิทยาลัยมหิดล เลขที่ 270 ถนนพระรามหก เขตราชเทวี กทม. 10400
6.4 นางสาวพัชรีย์ การสมบัติ	อยู่ที่ ภาควิชาพยาธิวิทยา คณะแพทยศาสตรรามาธิบดี มหาวิทยาลัยมหิดล เลขที่ 270 ถนนพระรามหก เขตราชเทวี กทม. 10400
6.5 นางสาวทัศนีย์ ฤกษ์สุทธิรัตน์	อยู่ที่ ภาควิชาพยาธิวิทยา คณะแพทยศาสตรรามาธิบดี มหาวิทยาลัยมหิดล เลขที่ 270 ถนนพระรามหก เขตราชเทวี กทม. 10400
6.6 แพทย์หญิงรังสิมา อรุณโรจน์	อยู่ที่ ภาควิชาพยาธิวิทยา คณะแพทยศาสตรรามาธิบดี มหาวิทยาลัยมหิดล เลขที่ 270 ถนนพระรามหก เขตราชเทวี กทม. 10400
6.7 นางทัศนีย์ โล้หนู	สำนักงานวิจัย คณะแพทยศาสตร์รามาธิบดี มหาวิทยาลัยมหิดล เลขที่ 270 ถนนพระรามหก เขตราชเทวี กทม. 10400
6.8 นางวันทา ยิ่งยง	สำนักงานวิจัย คณะแพทยศาสตร์รามาธิบดี มหาวิทยาลัยมหิดล เลขที่ 270 ถนนพระรามหก เขตราชเทวี กทม. 10400

PLOS | ONE

RESEARCH ARTICLE

The Elicitin-Like Glycoprotein, ELI025, Is Secreted by the Pathogenic Oomycete *Pythium insidiosum* and Evades Host Antibody Responses

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Data Availability Statement: All ELI025-coding sequences from P. insidiosum strain Pi-S, MCC18, and P01 have been submitted to the DNA data bank of Japan (DDBJ), under accession numbers AB971191 to AB971193.

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Abstract

Pythium insidiosum is a unique oomycete that can infect humans and animals. Patients with a *P. insidiosum* infection (pythiosis) have high rates of morbidity and mortality. The pathogen resists conventional antifungal drugs. Information on the biology and pathogenesis of *P. insidiosum* is limited. Many pathogens secrete proteins, known as effectors, which can affect the host response and promote the infection process. Elicitins are secretory proteins and are found only in the oomycetes, primarily in *Phytophthora* and *Pythium* species. In plant-pathogenic oomycetes, elicitins function as pathogen-associated molecular pattern molecules, sterol carriers, and plant defense stimulators. Recently, we reported a number of elicitin-encoding genes from the *P. insidiosum* transcriptome. The function of elicitins during human infections is unknown. One of the *P. insidiosum* elicitin-encoding genes, *ELI025*, is highly expressed and up-regulated at body temperature. This study aims to characterize the biochemical, immunological, and genetic properties of the elicitin protein, ELI025. A 12.4-kDa recombinant ELI025 protein (rELI025) was expressed in *Escherichia coli*. Rabbit anti-rELI025 antibodies reacted strongly with the native ELI025 in *P. insidiosum*'s culture medium. The detected ELI025 had two isoforms: glycosylated and non-glycosylated. ELI025 was not immunoreactive with sera from pythiosis patients. The region near the transcriptional start site of *ELI025* contained conserved oomycete core promoter elements. In conclusion, ELI025 is a small, abundant, secreted glycoprotein that evades host antibody responses. ELI025 is a promising candidate for development of diagnostic and therapeutic targets for pythiosis.

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Introduction

Pythium insidiosum is an organism that belongs to oomycetes, a group of fungus-like microorganisms [1]. While most of pathogenic oomycetes infect plants, *P. insidiosum* can infect humans and animals and cause a life threatening infectious disease, called pythiosis [1–4]. Although pythiosis is relatively rare compared to other infectious diseases, it has been increasingly reported from tropical and subtropical countries, such as, Brazil, Costa Rica, USA, Egypt, Mali, India, Malaysia, Thailand, Australia, and New Zealand [1–13]. Patients with pythiosis most commonly present with claudication and gangrenous ulcers of the lower extremities, as a result of chronic arterial infection and occlusive blood clots (vascular pythiosis) [4]. An alternative form, ocular pythiosis, presents with corneal ulcer and keratitis, as a result of ocular infection [4]. Pythiosis has a high rate of morbidity and mortality. Health care personnel often fail to recognize pythiosis, and this results in delayed diagnosis and contributes to the high mortality. Antifungal drugs are ineffective against *P. insidiosum*. Approximately, 80% of patients undergo surgical removal of the infected organ (leg or eye). In many advanced cases, surgery fails to eradicate the organism, and ~40% of the patient with vascular pythiosis die from the disease. Better understanding of the biology and pathogenesis of *P. insidiosum* could lead to discovery of new methods for prevention, diagnosis, and treatment of pythiosis.

Many pathogenic microorganisms secrete proteins that promote infection by interfering with host cell function and altering host responses [14–22]. For example, the bacterium *Helicobacter pyroli* secretes CagA to perturb a host cell signaling pathway, and leads to development of peptic ulcer [17,18]. The malarial parasite *Plasmodium falciparum* secretes some histidine-rich proteins that facilitate its survival inside red blood cells [19]. In many plant-pathogenic oomycetes, the multifunctional elicitin molecules facilitate infection by triggering host tissue necrosis [22]. The elicitin can also be recognized as a pathogen-associated molecular pattern by plant cells [23–26], and serve as a sterol-carrying protein for acquiring exogenous sterols [27–33].

Recent transcriptome analyses revealed that *P. insidiosum* harbors an extensive repertoire of elicitin-domain-containing proteins (~300 proteins), many of which (~60 proteins) are predicted to be secreted [34,35]. The biological role of elicitin in human hosts is unknown. The *P. insidiosum* elicitin-encoding gene, *ELI025*, is highly expressed and 5-fold up-regulated when *P. insidiosum* hyphae is grown at body temperature (37°C), compared to hyphae grown at room temperature (28°C) [34,35], suggesting that *ELI025* may be required for survival of *P. insidiosum* inside a human host. The current study reports on the cloning and expression of *ELI025* for genetic, biochemical and immunological analyses. Molecular characterization of elicitin is a significant step in exploring the biology and virulence of this understudied microorganism and could lead to new strategies for infection control.

Materials and Methods

Ethics statement

This study was approved, without requiring informed consent from patients, by the Committee on Human Rights Related to Research Involving Human Subjects, at the Faculty of Medicine, Ramathibodi Hospital, Mahidol University (approval number MURA2012/504S1). An informed consent was not obtained from patients (from whom microorganisms, tissues, and blood samples were obtained) because the data were analyzed anonymously.

Microorganisms

The *P. insidiosum* strains Pi-S, MCC18, and P01, were obtained from a collection of microorganisms that were isolated from clinical samples received for routinely culture identification.

All strains were maintained on Sabouraud dextrose agar at room temperature and sub-cultured once a month.

Serum samples

Three serum samples were obtained from pythiosis patients diagnosed by culture identification or serological tests [36–41]. To serve as controls, three serum samples were obtained from healthy blood donors who came to the Blood Bank Division, Department of Pathology, Ramathibodi Hospital. Rabbit anti-rELI025 sera were purchased from the Biomedical Technology Research Laboratory, Faculty of Associated Medicine, Chiang Mai University, Thailand. To block the rabbit anti-rELI025 antibodies from the rabbit serum, 20 μl of rELI025 (2.4 mg-/ml) and 1.5 ml of diluted rabbit serum [1:2,000 in 5% skim milk in TBS (pH 7.5)] were co-incubated with gentle agitation at 4°C overnight. All sera were kept at -20°C until use.

Protein preparation

Crude protein extracts of *P. insidiosum*, including soluble antigen from broken hyphae (SABH; containing intracellular proteins) and culture filtrate antigen (CFA; containing secreted proteins), were prepared according to the methods described by Chareonsirisuthigul et al [41]. Briefly, 100 ml Sabouraud dextrose broth was inoculated from an actively growing *P. insidiosum* colony and incubated, with shaking (~150 rpm), at 37°C for 10 days. The organism was killed with 0.02% Thimerosol (Sigma). Hyphae were collected by filtration on a 0.22-μm-pore-size membrane (Durapore, Merck Millipore), and ground in a mortar with pre-cooled distilled water (1.5 g hyphae per 30 ml water). Supernatant, following centrifugation (10,000 x g) of the cell lysate at 4°C for 30 min, was filtered through a 0.22-μm-pore-size membrane (Durapore, Merck Millipore). Both filtered supernatant (SABH) and cell-free broth (CFA) were 100-fold concentrated by ultrafiltration (10,000 molecular weight cut-off membrane; Amicon Ultra 15M, Merck Millipore). Protein concentration was measured by Bradford's assay [42]. SABH and CFA were stored at -20°C until use.

Genomic DNA extraction

P. insidiosum genomic DNA (gDNA) was extracted using the modified method of Lohnoo et al [43]. Briefly, hyphal mat (~500 mg) was transferred to a 2-ml tube containing glass beads (~1,000-μm diameter; Sigma) and 400 μl of homogenizing buffer [0.4 M NaCl, 10 mM Tris—HCl (pH 8.0), 2 mM EDTA (pH 8.0)]. The tube was shaken at 30 Hz for 2 min, in a tissue homogenizer (TissueLyzer, QIAGEN), before adding 20% sodium dodecyl sulfate (final concentration, 2%) and proteinase K (final concentration, 400 μg/ml). The cell lysate was incubated, with gentle inversion, at 55°C, overnight. The sample was then mixed with 300 μl of 6 M NaCl, vigorously vortexed for 30 s, and centrifuged (10,000 x g) at room temperature for 30 min. The supernatant was then mixed with an equal volume of isopropanol, incubated at -20°C for 1 hr, and centrifuged (12,000 x g) at 4°C for 20 min. The gDNA pellet was collected and washed with 70% ethanol, air dried, and resuspended in sterile water. All extracted gDNAs were kept at -20°C until use.

Plasmid construction

The full-length ELI025-encoding sequence (NCBI accession number: HS975204) was amplified from the pCR4-blunt-TOPO vector harboring PinsEST#025 cDNA [34], in a 50-μl PCR reaction containing 1.5 μl of PCR product, 1 μl of the Elongase and its buffer mixture (buffer A:B ratio = 1:4) (Invitrogen), 200 μM of dNTPs, and 0.4 μM each of the primer

ELI025_NdeI (5′ -GGCATCACATATGtacaacgagaccaagccg-3') and ELI025_EcoRI (5′ -CAAGAATTCCTAGGCCTTGCAGCTCGTC-3'). The reaction was carried out in a MyCycler (Biorad) with the following conditions: initial denaturation at 94°C for 30 s, 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 68°C for 1.10 min, and final extension at 68°C for 5 min. The PCR product was double digested with *NdeI* and *EcoRI* (New England Biolabs), and directionally cloned into pET28b (Novagen), yielding an in-frame His-tag fusion on the N-terminus of ELI025. The resulting plasmid, pET28b-ELI025 (Fig. 1A), was propagated in the *Escherichia coli* strain DH5α. The sequence of the ELI025-coding region of the plasmid was confirmed using primers, T7-promoter (5′ -TAATACGACTCACTA-TAGGG-3') and T7-terminator (5′ -GCTAGTTATTGCTCAGCGG-3').

Protein expression and purification

The recombinant ELI025 protein (rELI025; plasmid pET28b-ELI025) was expressed from the *E. coli* strain rosetta-gami2 (DE3) (Novagen). A clone harboring pET28b-ELI025 was grown in the Terrific broth [44], supplemented with tetracycline (12.5 μg/ml), chloramphenicol (34 μg-/ml), and kanamycin (30 μg/ml), until the cells reached 0.5 optical density. IPTG (final

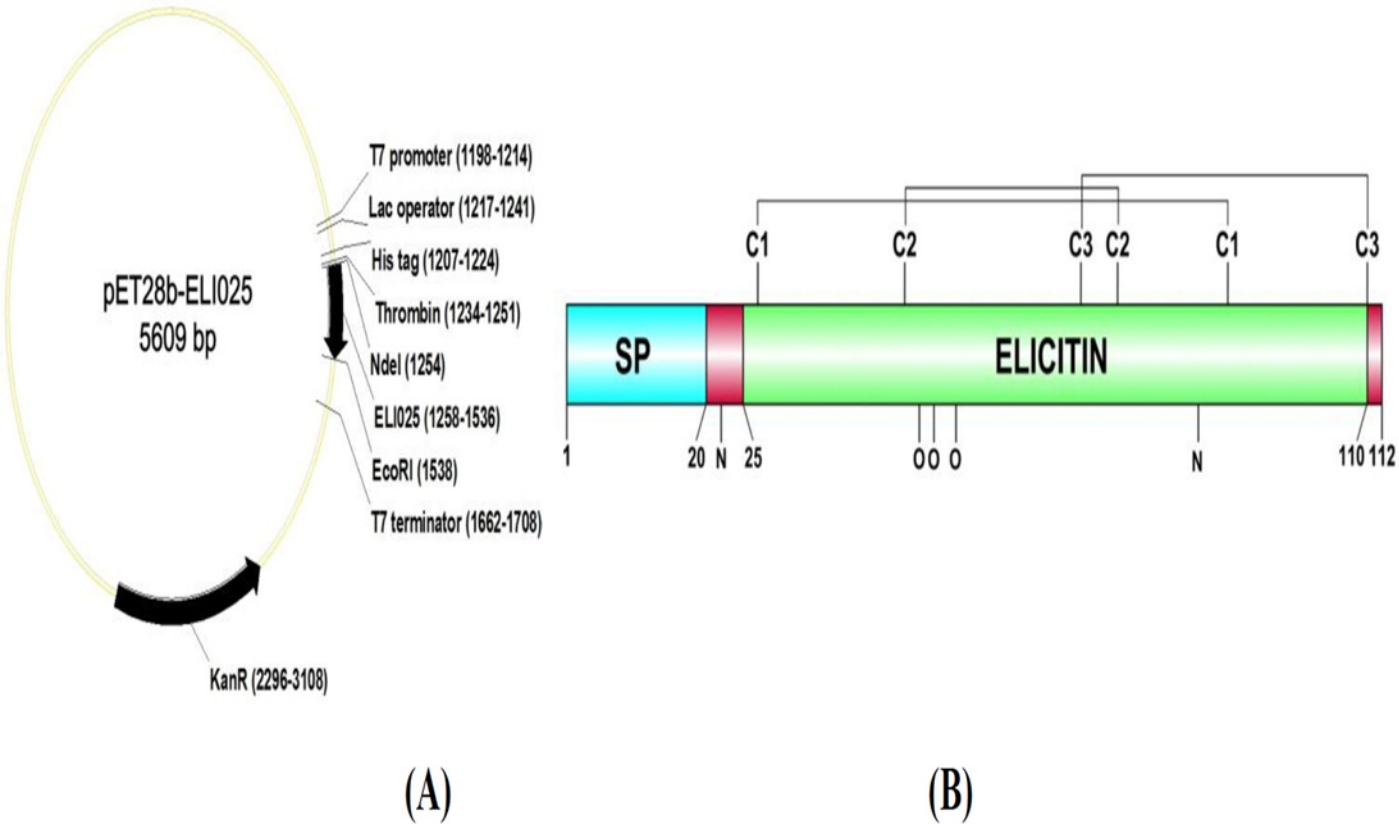

Fig 1. Cloning and expression of *ELI025*. (**A**) Plasmid DNA map of pET28b-ELI025 shows the cloning sites (*Nde*-I and *EcoR*-I) of *ELI025*. Expression of *ELI025* is under the control of the T7 promoter. The numbers in parentheses indicate a location of each plasmid component; (**B**) Protein structure of ELI025 shows a signal peptide (SP; amino acid position 1–20), an elicitin domain (amino acid position 25–110), three disulfide bonds (C1, cysteine position 27 and 91; C2, cysteine position 47 and 76; C3, cysteine position 71 and 110), two predicted N-linked glycosylation sties (N; amino acid position, 22 and 87), and three predicted O-linked glycosylation sties (O; amino acid position 49, 51, and 54).

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concentration, 1 mM; Omnipur) was added, before further shaking incubation (250 rpm) at 25°C for 12 hr. The culture was centrifuged (6000 x g) at 4°C for 10 min and the pellet was resuspended in binding buffer [20 mM sodium phosphate buffer (pH 7.4) and 0.1 M NaCl] (1 g pellet per 5 ml binding buffer), mixed with lysozyme (final concentration, 1 mg/ml; BioBasics), incubated on ice for 30 min, sonicated (setting: 50% amplitude, 20 cycles, 10/10-second pulse on/off), and centrifuged (10,000 x g) at 4°C for 30 min. The resulting supernatant was applied to a HiTrap IMAC FF column (GE healthcare), pre-charged with 0.1 M $NiCl_2$. The column was sequentially washed with the binding buffer containing 60 and 100 mM imidazole. Protein was eluted from the column with binding buffer containing 500 mM imidazole. The concentration of the purified recombinant protein was determined by Bradford's assay [42], and kept at -30°C until use.

SDS-PAGE and Western blot

SABH, CFA, and rELI025 were separated by SDS-PAGE (4% stacking and 12% separating gel) at 150 V, for 65 min, using the Mini-PROTEAN II apparatus (Biorad). Proteins were stained with either Coomassie blue R-250 or Silver staining kit (Thermo Scientific). The Image Lab 3.0 program (Biorad) was used to estimate protein molecular weight (kilo Dalton; kDa) based on migration of pre-stained broad range protein markers (Biorad). For Western blot analysis, the separated proteins were transferred and immobilized onto a 0.2-μm-pore-size PVDF membrane (Merck Millipore), using the Biorad Mini Trans-Blot cell (setting: 100 V for 60 min). The blotted membrane was blocked with 5% skim milk (Sigma) in blocking buffer [TBS; 150 mM NaCl, 10 mM Tris-HCl (pH 7.5)] at 4°C, overnight, or room temperature for an hour. The membrane was washed 3 times with the washing buffer [TTBS; 500 mM NaCl, 20 mM Tris-Cl, and 0.1% Tween-20 (pH 7.5)]. The membranes were incubated with the primary antibodies diluted in the blocking buffer [1:1,000 for mouse anti-6x histidine antibody (Merck Millipore); 1:2,000 for rabbit anti-rELI025 serum; 1:1,000 for patient serum] for 2 hr at room temperature (anti-6x histidine and anti-rELI025) or overnight at 4°C (patient serum), and washed 3 times with TTBS. The secondary antibodies, diluted in the blocking buffer [1:2,000 for goat anti-mouse IgG conjugated with horseradish peroxidase (Merck Millipore); 1:5000 for goat anti-rabbit IgG conjugated with alkaline phosphatase (Southern Biotech); 1:3000 for goat anti-human IgG conjugated with horseradish peroxidase (Biorad)], were added to the membrane and incubated for 2–3 hr at room temperature. After washing the membrane 3 times with TTBS, substrate and chromogen (4CN and H_2O_2 for horseradish peroxidase; NBT and BCIP for alkaline phosphatase) were added to develop Western blot signals. Intensities of Western blot bands were quantitated by the GelQuant.NET software (http://biochemlabsolutions.com/GelQuantNET.html).

Mass spectrometric analysis

The 10- and 15-kDa bands present on SDS-PAGE gel and Western blot were excised from gel and PVDF membrane, respectively. Proteins were extracted and trypsin digested, using the method described by Shevchenko et al. [45]. The digested proteins were analyzed by an Ultimate 3000 LC System (Dionex, USA) coupled to an HCTultra PTM Discovery System (Bruker Daltonics Ltd., U.K.) at the Proteomics Research Laboratory, Genome Institute, National Center for Genetic Engineering and Biotechnology, Thailand. The Bruker Daltonics Data Analysis version 4.0 (Bruker Daltonics Ltd., U.K.) was used to analyze raw mass spectrometric data. The MASCOT software (Matrix Science, UK) was used to search the obtained MS and MS/MS data against ~15,000 genome-derived predicted proteins of *P. insidiosum* (unpublished data).

Deglycosylation of glycoprotein

A glycoprotein deglycosylation kit (Calbiochem) was used to remove sugar moieties (N- and O-linked glycosylation) from the native ELI025 in CFA. Briefly, 50 μg of CFA, 10 μl of 5x kit deglycosylation buffer, 2.5 μl of the kit denaturation solution, and distilled water were mixed to the final volume of 50 μl. The mixture was heated at 100°C for 5 min and cooled down to room temperature, before adding 2.5 μl of 15% TRITON X-100. To remove N-glycosyl groups, 1 μl of N-glycosidase F was added to the reaction. To remove O-glycosyl groups, 1 μl of the enzyme mixture, including α-2–3,6,8,9-neuraminidase, endo-α-N-acetylgalactosaminidase, β1,4-galactosidase, and β-N-acetylglucosaminidase, was added to the reaction. The protein-enzyme mixture was incubated at 37°C for 3 hr.

Immunohistochemical staining assay

Immunohistochemical staining assay was performed, using the method described by Keeratijarut *et al* [46], with some modifications. A paraffin-embedded tissue section (4-μm thickness) from a patient with vascular pythiosis was pretreated with xylene and absolute ethanol (Merck), before washing with phosphate buffered saline (PBS; pH 7.4). The tissue section was incubated with Tris-EDTA buffer (TE buffer; pH 9.0) at 95°C for 40 min, treated with 10% H_2O_2 in PBS for 10 min, and washed with PBS. Then, the tissue section was incubated with 200 μl of either rabbit pre-immune serum or rabbit anti-rELI025 serum (1:16,000 in PBS) at 4°C overnight, washed with PBS (5 min each), and incubated with 200 μl of mouse anti-rabbit IgG antibody conjugated with horseradish-peroxidase (Thermo Scientific, USA) for 30 min. To develop color, the substrate 3,3'-diaminobenzidine tetrahydrochloride (DAKO, USA) was added to the tissue section and incubated at room temperature for 5 min. The tissue section was counterstained with hematoxylin before examination with a light microscope (ECLIPSE Ci, Nikon, Japan).

Polymerase chain reaction and DNA sequencing

A draft genome sequence of the *P. insidiosum* strain Pi-S (unpublished data) was used to design primers for PCR amplification of the rELI025-encoding sequence and its promoter region (ELI025_promoter_F2, 5′ -CATGGACAGCGTCATCTCTGG-3'; ELI025_promoter_R1, 5′ -GCGTCAAGATGAGAAACGAGG-3'). Each amplification reaction was performed in a 50-μl reaction containing 100 ng of genomic DNA template, 0.02 U/μl of DNA polymerase (Phusion), 1x Phusion buffer, 200 μM of dNTPs, and 0.5 μM each of the primer. The amplification was carried out in a Mastercycler Nexus thermal cycler (Eppendorf), with the following conditions: an initial denaturation at 98°C for 30 s, 35 cycles of denaturation at 98°C for 10 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min, and a final extension at 72°C for 10 min. The PCR products were purified using a NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel) and assessed for amount and size by 1% gel electrophoresis.

Direct sequencing of PCR products was performed using a BigDye terminator V3.1 cycle sequencing kit (Applied Biosystems) and an ABI Prism 3100 Genetic Analyzer (Applied Biosystems). The primers used for sequencing included ELI025_F1 (5′ -TACAACGAGAC-CAAGCCGTG-3'), ELI025_R1 (5′ -GGCCTTGCAGCTCGTCTC-3'), ELI025_promoter_F2, ELI025_promoter_R1, ELI025_promoter_seqF1 (5′ -CGCCCCCTTTCTTCCCGAC-3') and ELI025_promoter_seqR1 (5′ -CCAACCAGACGCCGTCTG-3'). The sequences were analyzed using the ABI Prism DNA Sequencing Analysis software (Applied Biosystems, USA).

Bioinformatic analysis

The molecular weight of ELI025 was calculated by ProtParam [47]. Signal peptide, transmembrane domain, N- and O-linked glycosylation and GPI-anchor of ELI025 were predicted using the SignalIP program version 4.0 [48], the TMHMM program version [49], the NetNGlyc (http://www.cbs.dtu.dk/services/NetNGlyc), and NetOGlyc [50] programs, and the big-PI predictor [51], respectively. The promoter and ELI025-coding sequences from all *P. insidiosum* strains used in this study were aligned and compared using the ClustalX program version 2 and the GeneDoc program [52,53].

Homologous protein search

The elicitin domain sequence of ELI025 was used to BLAST search for elicitin homologous proteins encoded in the genomes and transcriptomes, or present in the proteomes of 18 oomycetes, 10 fungi, 4 algae, 3 diatoms, and one protozoan [54–72] (Table 1). The cut-off E-value for BLAST searches was $\leq 1 \times 10^{-4}$. If a BLAST search of particular genome database was not possible online, then a local BLASTP and TBLASTN search was performed using the BLAST 2.2.28+ program (http://www.ncbi.nlm.nih.gov/news/04-05-2013-blast-2-2-28/).

Phylogenetic analysis

Elicitin domain sequences from different oomycete organisms (Table 1) were analyzed online at http://www.phylogeny.fr/ [73]. The sequences were aligned by MUSCLE [74], and phylogenetic relationships were calculated by Neighbor-joining with 1,000 bootstraps [75] and the Jones-Taylor-Thornton matrix substitution model [76]. A phylogenetic tree was generated by TreeDyn [77].

Nucleotide sequence accession numbers

All ELI025-coding sequences from *P. insidiosum* strain Pi-S, MCC18, and P01 have been submitted to the DNA data bank of Japan (DDBJ), under accession numbers AB971191 to AB971193, respectively.

Results

Structures of *ELI025* and its gene product

The DNA sequence covering the 5'-untranslated region, coding sequence, and 3'-untranslated region of the *ELI025* gene was successfully PCR-amplified from gDNA of three different *P. insidiosum* strains: Pi-S (1,106-bp long; accession number, AB971191), MCC18 (1,056-bp long; accession number, AB971192), and P01 (1,036-bp long; accession number, AB971193). No intron was identified when the gDNA-derived (accession number, AB971191–3) and mRNA-derived (accession number, HS975204 and FX528334) ELI025-coding sequences were aligned. Analyses of the coding sequences for the *ELI025* alleles of three different strains of *P. insidiosum* by ClustalX version 2 [52] and GeneDoc [53] programs showed 98–99% identity and 99–100% similarity with each other (data not shown).

The 5'-untranslated and -flanking DNA sequences of the *ELI025* gene from the three *P. insidiosum* strains were compared with that of various genes from several oomycetes and parasites (Fig. 2). These sequences share a 19-nucleotide oomycete core-promoter sequence, located between 9 and 79 nucleotides upstream of the start codon (Fig. 2). Two putative core-promoter components, an initiator element (Inr; 5'-TCATTCC-3') and a flanking promoter region (FPR; 5′ -CAACCTTCC-3'), were identified in this region of *ELI025* (Fig. 2). A predicted

Table 1. BLAST search of the ELI025 amino acid sequence against the genomes, transcriptomes, or proteomes of 18 oomycetes, 10 fungi, 4 algae, 3 diatoms, and one protozoan (the cut-off E-value ≤ 1 x 10–4).

Organisms	Group	Subgroup	Number of BLAST hits	E-value of the best BLAST hit	References
Pythium ultimum	Oomycete	Pythiales	27	3.30E-31	[54]
Pythium aphanidermatum	Oomycete	Pythiales	20	1.00E-15	[54]
Pythium irregulare	Oomycete	Pythiales	18	2.00E-34	[54]
Pythium arrhenomanes	Oomycete	Pythiales	17	8.80E-16	[54]
Pythium iwayamai	Oomycete	Pythiales	14	7.00E-30	[54]
Pythium vexans	Oomycete	Pythiales	14	1.00E-20	[54]
Phytophthora sojae	Oomycete	Peronosporales	26	1.46E-22	[55]
Phytophthora ramorum	Oomycete	Peronosporales	25	1.00E-21	[55]
Phytophthora parasitica	Oomycete	Peronosporales	16	1.11E-14	BI[a]
Phytophthora capsici	Oomycete	Peronosporales	15	7.02E-20	[56]
Phytophthora cinnamomi	Oomycete	Peronosporales	14	5.51E-20	JGI[b]
Phytophthora infestans	Oomycete	Peronosporales	10	2.11E-16	BI[a]
Pseudoperonospora cubensis	Oomycete	Peronosporales	6	2.00E-07	[57]
Hyaloperonospora arabidopsis	Oomycete	Peronosporales	2	1.00E-06	[58]
Albugo laibachii	Oomycete	Albuginales	1	6.40E-13	[59]
Aphanomyces euteiches	Oomycete	Saprolegniales	-	-	[60]
Saprolegnia diclina	Oomycete	Saprolegniales	-	-	BI[a]
Saprolegnia parasitica	Oomycete	Saprolegniales	-	-	[61]
Phaeodactylum tricornutum	Diatom	Bacillariophyta	-	-	[62]
Pseudo-nitzschia multiseries	Diatom	Bacillariophyta	-	-	JGI[b]
Thalassiosira pseudonana	Diatom	Bacillariophyta	-	-	[63]
Aurantiochytrium limacinum	Microalgae	Labyrinthulida	-	-	JGI[b]
Nannochloropsis gaditana	Microalgae	Eustigmatophyceae	-	-	[64]
Aureococcus anophagefferens	Brown tide algae	Pelagophyceae	-	-	[65]
Ectocarpus siliculosus	Brown algae	PX clade	-	-	[66]
Blastocystis hominis	Protozoan	Blastocystis	-	-	[67]
Aspergillus spp.	Fungi	Ascomycota	-	-	[68]
Candida spp.	Fungi	Ascomycota	-	-	[69]
Fusarium oxysporum	Fungi	Ascomycota	-	-	BI[a]
Histoplasma capsulatum	Fungi	Ascomycota	-	-	BI[a]
Paracoccidioides brasiliensis	Fungi	Ascomycota	-	-	BI[a]
Pneumocystis jirovecii	Fungi	Ascomycota	-	-	[70]
Mucor circinelloides	Fungi	Zygomycota	-	-	BI[a]
Rhizopus delemar	Fungi	Zygomycota	-	-	BI[a]
Rhizopus oryzae	Fungi	Zygomycota	-	-	[71]
Cryptococcus neoformans	Fungi	Basidiomycota	-	-	[72]

[a] Broad institute genome database

[b] Genome portal of the Department of Energy Joint Genome Institute

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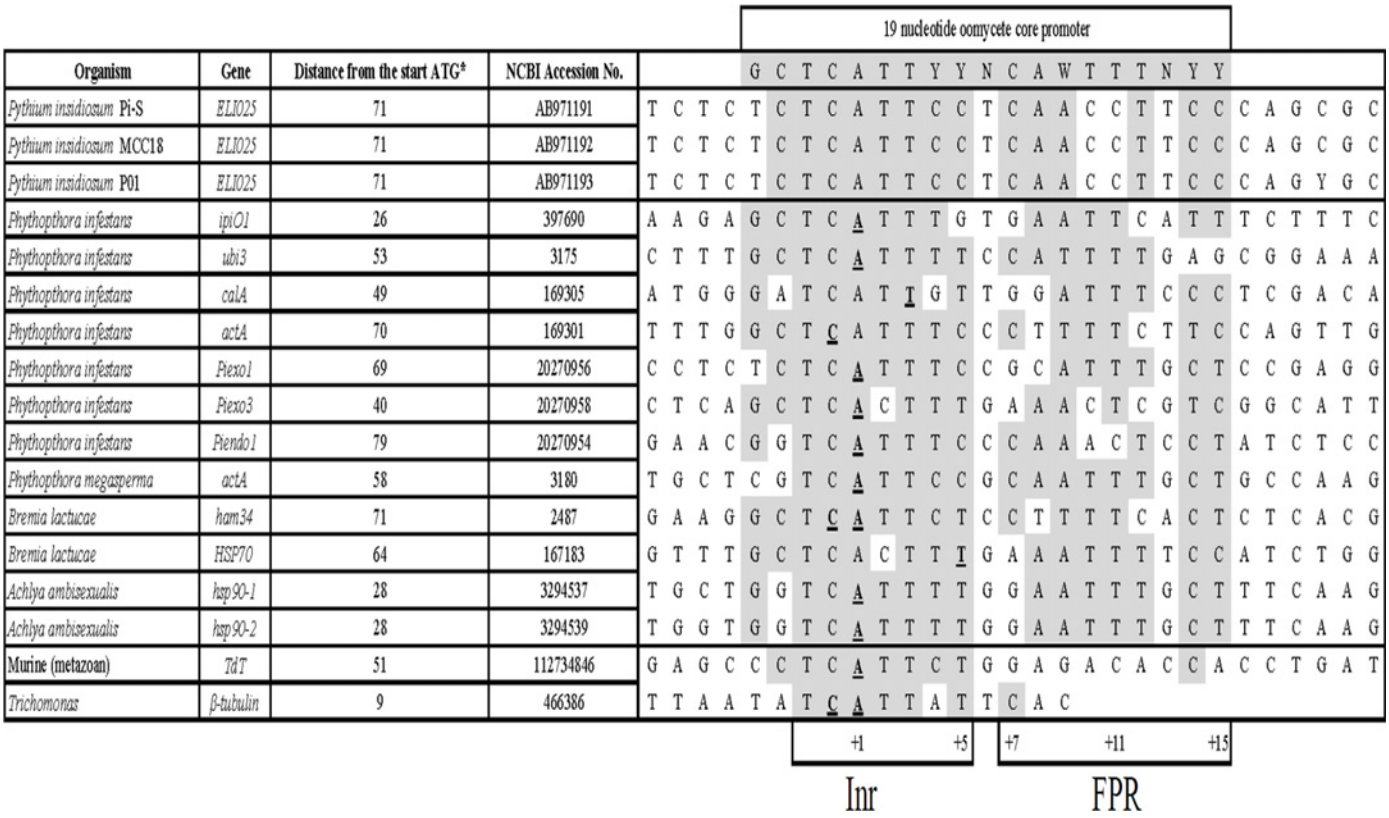
transcription start site (+1; Fig. 2) of the *ELI025* gene is within the Inr element. A TATA box was not observed in the promoter region of *ELI025*.

The predicted full-length ELI025 protein sequences (112 amino acids long) from the three *P. insidiosum* strains were 100% identical. A predicted signal peptide of ELI025 covered the first 20 N-terminal amino acids (Fig. 1B). The calculated molecular weights of ELI025, with and without the signal peptide, were 12 and 10 kDa, respectively. The elicitin domain spanned from amino



Organism	Gene	Distance from the start ATG*	NCBI Accession No.	19 nucleotide oomycete core promoter
				G C T C A T T Y Y N C A W T T T N Y Y
Pythium insidiosum Pi-S	*ELI025*	71	AB971191	T C T C T C T C A T T C C T C A A C C T T C C C A G C G C
Pythium insidiosum MCC18	*ELI025*	71	AB971192	T C T C T C T C A T T C C T C A A C C T T C C C A G C G C
Pythium insidiosum P01	*ELI025*	71	AB971193	T C T C T C T C A T T C C T C A A C C T T C C C A G Y G C
Phythopthora infestans	*ipiO1*	26	397690	A A G A G C T C A T T T G T G A A T T C A T T T C T T T C
Phythopthora infestans	*ubi3*	53	3175	C T T T G C T C A T T T T C C A T T T T G A G C G G A A A
Phythopthora infestans	*calA*	49	169305	A T G G G A T C A T T G T T G G A T T T C C C T C G A C A
Phythopthora infestans	*actA*	70	169301	T T T G G C T C A T T T C C C T T T T C T T C C A G T T G
Phythopthora infestans	*Piexo1*	69	20270956	C C T C T C T C A T T T C C G C A T T T G C T C C G A G G
Phythopthora infestans	*Piexo3*	40	20270958	C T C A G C T C A C T T T G A A A C T C G T C G G C A T T
Phythopthora infestans	*Piendo1*	79	20270954	G A A C G G T C A T T T C C C A A A C T C C T A T C T C C
Phythopthora megasperma	*actA*	58	3180	T G C T C G T C A T T C C G C A A T T T G C T G C C A A G
Bremia lactucae	*ham34*	71	2487	G A A G G C T C A T T C T C C T T T T C A C T C T C A C G
Bremia lactucae	*HSP70*	64	167183	G T T T G C T C A C T T T G A A A T T T T C C A T C T G G
Achlya ambisexualis	*hsp90-1*	28	3294537	T G C T G G T C A T T T T G G A A T T T G C T T T C A A G
Achlya ambisexualis	*hsp90-2*	28	3294539	T G G T G G T C A T T T T G G A A T T T G C T T T C A A G
Murine (metazoan)	*Tdt*	51	112734846	G A G C C C T C A T T C T G G A G A C A C C A C C T G A T
Trichomonas	*β-tubulin*	9	466386	T T A A T A T C A T T A T T C A C
				+1 +5 +7 +11 +15
				Inr FPR

Fig 2. Sequence alignment of core promoter regions of the *P. insidiosum ELI025* gene and various genes from several oomycetes and parasites. The *ELI025* sequences (accession number AB971191–3), used for the alignment, are derived from three different *P. insidiosum* strains. Conserved nucleotides are highlighted in grey. The underlined letters indicate the known transcriptional start site, and is indicated below as "+1". Two putative core promoter components, an initiator element (Inr; 5'-TCATTCC-3'; positions-2 to +5) and a flanking promoter region (FPR; 5′ -CAACCTTCC-3'; positions +7 to +15), are found in the upstream region of all genes. (Abbreviation: NCBI, National Center for Biotechnology Information).

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acid position 25 to 110. By analogy to known elicitins [34], three disulfide bonds (Cys27/Cys91, Cys47/Cys76, and Cys71/Cys110) were identified within the elicitin domain of ELI025 (C1, C2, C3; Fig. 1B). There are two predicted N-linked glycosylation sites at amino acid positions 22 and 87, and three predicted O-linked glycosylation sites at amino acid positions 49, 51, and 54 (Fig. 1B). Neither a GPI anchor nor a transmembrane region are predicted for ELI025.

Homologous proteins of ELI025

The ELI025 amino acid sequence was used for BLAST analyses of the genomes, transcriptomes, and proteomes of 36 different microorganisms (Table 1). No significant BLAST hit was identified in non-oomycete organisms. Three oomycetes (*A. euteiches*, *S. diclina*, and *S. parasitica*), which belong to the subgroup Saprolegniales, lacked sequences homologous to ELI025. A number of BLAST hits were found in 15 species of oomycetes, including *Pythium* spp. (14–27 hits), *Phytophthora* spp. (10–26 hits), *P. cubensis* (6 hits), *H. arabidopsis* (2 hits), and *A. laibachii* (1 hit). A signal peptide and an elicitin domain were identified in these top BLAST hit proteins (Fig. 3A). The similarity between the ELI025 signal peptide and signal peptides of the other oomycetes' elicitins (17–23 amino acids long) was high (mean, 44%; median, 47%; range, 19–71%). Elicitin domain sequences of ELI025 and the other top BLAST hit proteins (83–94 amino acids long) contained 6 conserved cysteine residues (Fig. 3A). Phylogenetic analysis,

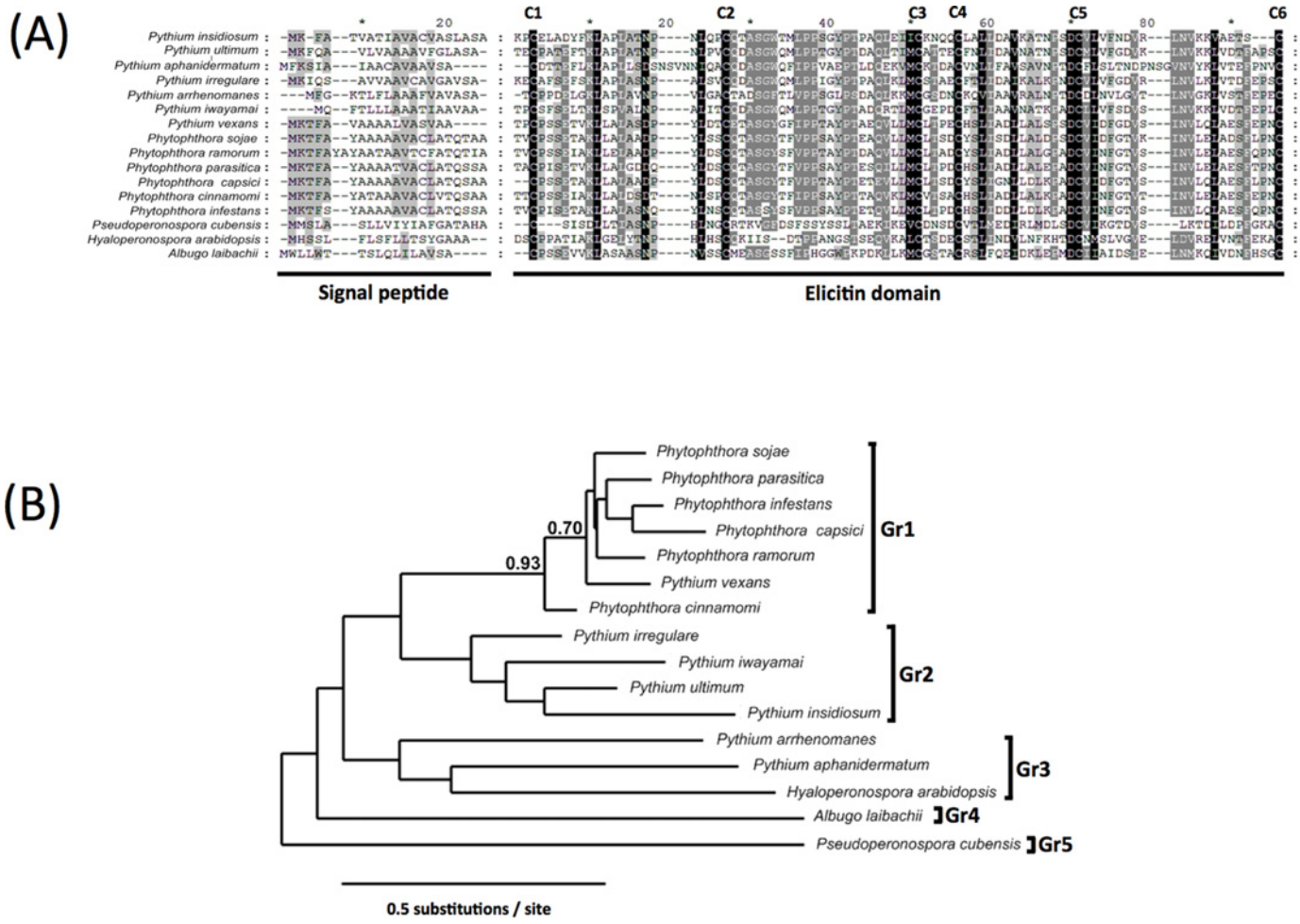

Fig 3. Sequence alignment and phylogenetic analysis of elicitin proteins. (**A**) Signal peptide (length, 17–23 amino acids) and elicitin domain (length, 83–94 amino acids) sequences of *P. insidiosum* ELI025 and the top BLAST hit proteins of 15 other oomycetes (Table 1) were aligned and compared. C1–C6 indicate conserved cysteine residues; (**B**) Phylogenetic analysis of elicitins by the neighbor-joining method. The phylogenetic tree, constructed from elicitin domain sequences of *P. insidiosum* ELI025 and the top BLAST hit proteins of 15 other oomycetes (Table 1), shows three major clades (as indicated by Gr1, Gr2 and Gr3; containing multiple sequences per clade) and two minor clades (as indicated by Gr4 and Gr5; containing one sequence per clade). Only the branch support values of 70% or more are shown at corresponding nodes.

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based on the elicitin domain sequences, divided the oomycetes into 5 closely related groups (Gr1–5): Gr1 contained all *Phytophthora* species; Gr2 and Gr3 comprised mainly *Pythium* species; Gr4 included *A. laibachii* and Gr5 had only *P. cubensis* (Fig. 3B).

ELI025 is a major secreted non-immunogenic glycoprotein

The recombinant protein, rELI025, was successfully expressed and purified from *E. coli* (protein yield: 2 mg per 1 liter of bacterial culture). Purity of rELI025 was at least 99%, as demonstrated by silver staining analysis of SDS-PAGE gel. The molecular weight of rELI025 in the SDS-PAGE gel was estimated to be 12.4 kDa (Fig. 4A). rELI025 appeared as an intense 12.4-kDa Western blot band, when reacted with the mouse anti-6x histidine-tag antibody (data not shown) or the rabbit anti-rELI025 antibodies (Fig. 4B).

Gel-separated total proteins from crude extracts or supernatants of three *P. insidiosum* strains, had molecular weights ranging from 6 to 115 kDa (Fig. 4A). In Western Blots, rabbit

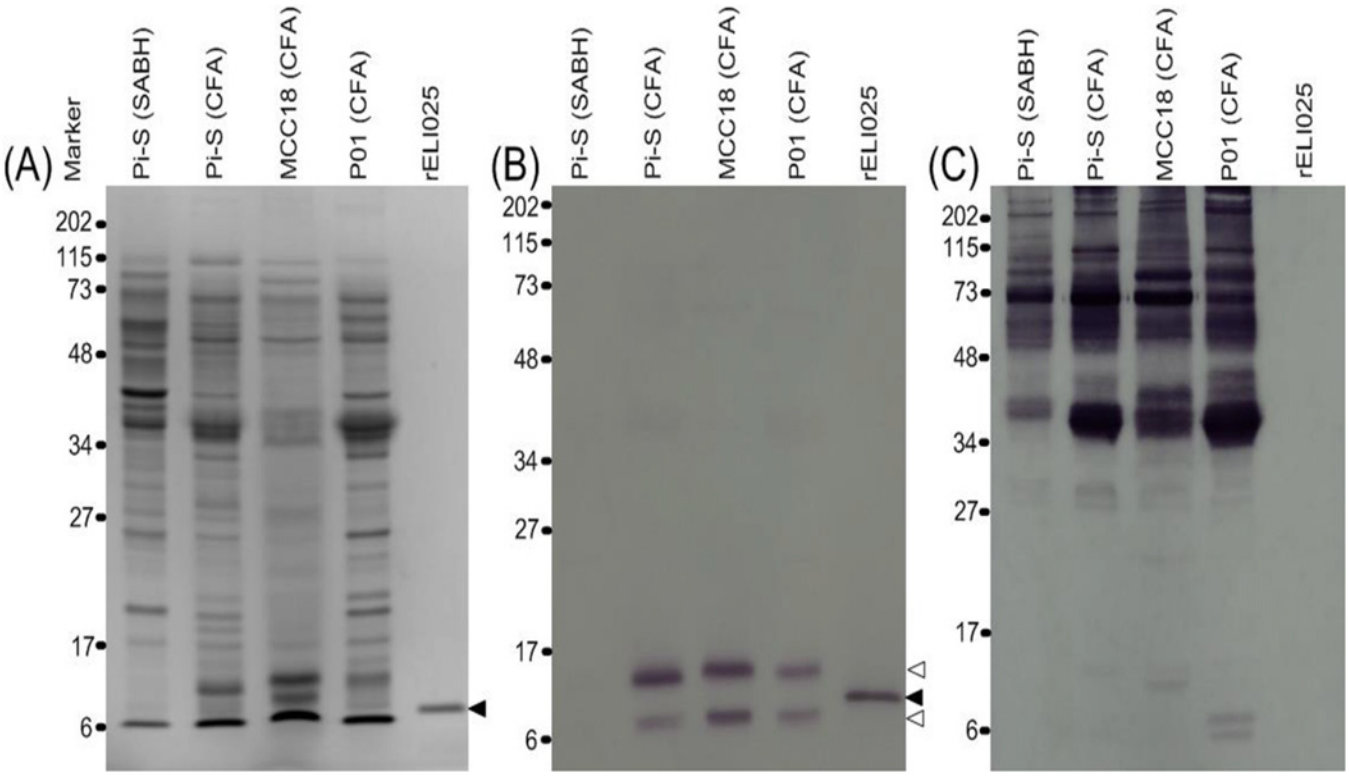

Fig 4. Immunoreactivity of the recombinant protein rELI025 and crude protein extracts of *P. insidiosum*. Crude proteins (i.e., SABH and CFA) extracted from three different strains of *P. insidiosum* (Pi-S, MCC18, and P01) and rELI025 are separated in a SDS-PAGE gel **(A)**. The separated proteins are analyzed by Western blot, using the rabbit anti-rELI025 antibodies **(B)**, or sera from patients with pythiosis **(C)**, as probe. The black arrow head indicates the 12.4 kDa band of rELI025. The white arrow heads indicate the 10- and 15-kDa bands of native ELI025. The numbers represent protein molecular weights standards, in kDa. (Abbreviations: SDS-PAGE, Sodium dodecyl sulfate polyacrylamide gel electrophoresis; CFA, culture filtrate antigen; SABH, soluble antigen from broken hyphae; rELI025, recombinant ELI025).

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anti-rELI025 serum reacted only with the 10- and 15-kDa bands in CFA (culture filtrate antigen), which contains secreted proteins of *P. insidiosum* (Fig. 4B). The rabbit anti-rELI025 serum did not react any proteins in SABH (soluble antigens from broken hyphae), which contains intracellular proteins (Fig. 4B). The rabbit pre-immune serum did not detect any proteins in SABH or CFA. If the rabbit anti-rELI025 serum is pre-absorbed with rELI025 protein prior to Western Blot detection, the band intensities for the 10- and 15-kDa proteins were reduced by ~85% (data not shown).

The native ELI025 (nELI025) in CFA was treated with protein deglycosylases to remove either N- or O-linked glycosyl adducts. The SDS-PAGE and Western blot profiles (probed with the rabbit anti-rELI025 serum) show that the 15-kDa band disappears in the CFA treated with the N-linked deglycosylation enzyme (either alone or in combination with the O-linked deglycosylase; Fig. 5). In contrast, the 10- and 15-kDa bands were both present in CFA treated with O-linked deglycosylase or in the no enzyme control.

The 10- and 15-kDa bands excised from SDS-PAGE gel (Fig. 3A) and Western blot membrane (Fig. 3B) were analyzed by LC-MS/MS (see Methods). MASCOT analysis of MS data showed that the 10-kDa SDS-PAGE band-derived peptides with mass-to-charge ratio (m/z) of 569.6, 686.9 and 853.9, and the 15-kDa SDS-PAGE band-derived peptides with m/z of 458.3, 569.6, 686.9 and 853.9, matched the ELI025 protein in the *P. insidiosum*'s proteome (Fig. 6A; Table 2). No peptide mass of the 10- and 15-kDa band excised from Western blots matched ELI025. Further MASCOT analyses of MS/MS data of the 10- and 15-kDa SDS-PAGE band-

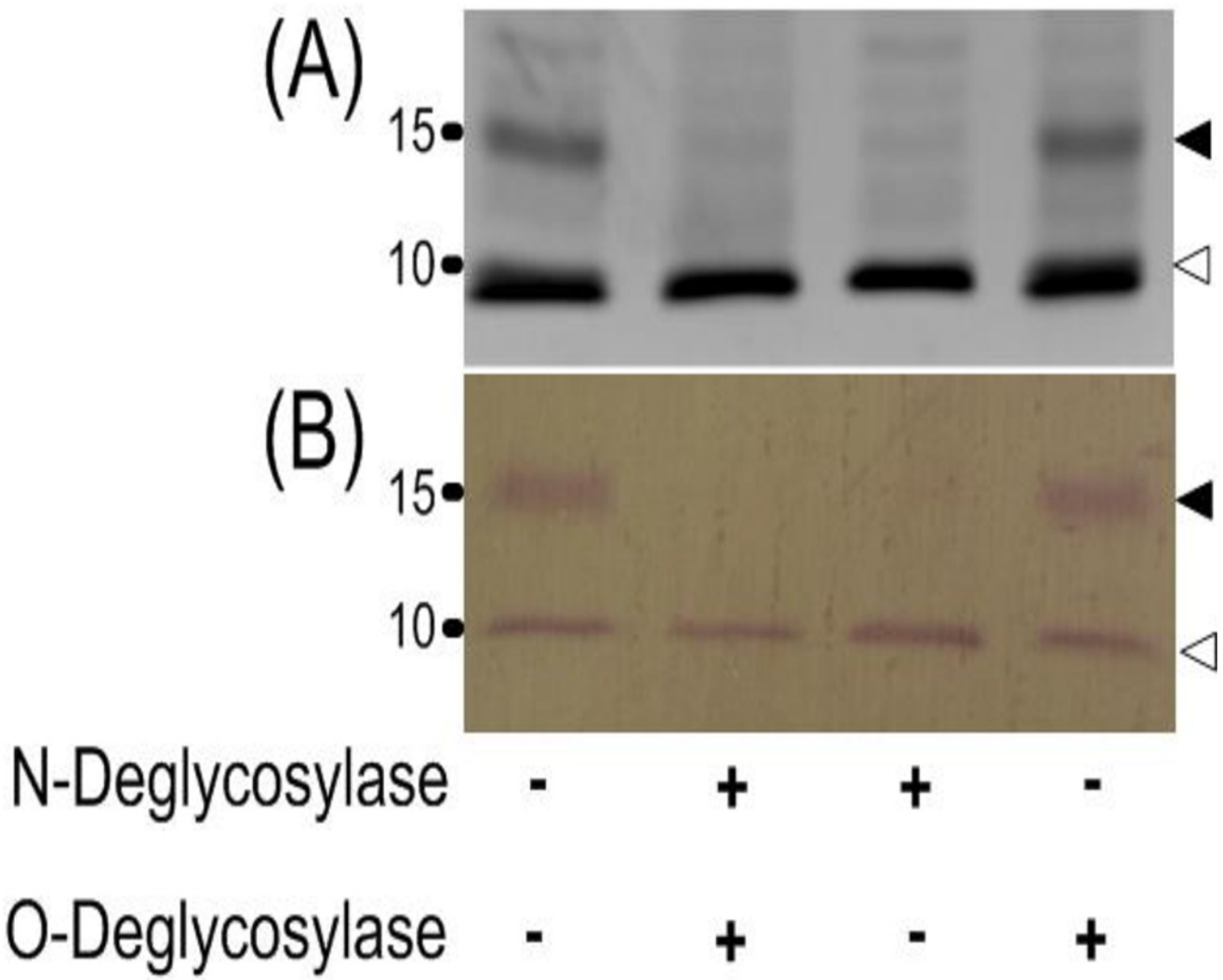

Fig 5. Protein deglycosylation of ELI025. CFA proteins were untreated (control; Lane 1) or treated with either N-Deglycosylase (N-glycosidase F; Lane 3), or O-Deglycosylase (a mixture of α-2–3,6,8,9-neuraminidase, endo-α-N-acetylgalactosaminidase, β-1,4-galactosidase, and β-N-acetylglucosaminidase; Lane 4), or both N- and O-Deglycosylases (Lane 2). The enzyme-treated proteins were separated on a SDS-PAGE gel **(A)**, and were further analyzed by Western blot, using the rabbit anti-rELI025 antibodies as primary antibody **(B)**. Only the low molecular weight portion of the gel and blot are shown. The black and white arrow heads point out the 15-kDa and 10-kDa bands. (Abbreviations: SDS-PAGE, Sodium dodecyl sulfate polyacrylamide gel electrophoresis; CFA, culture filtrateantigen; rELI025, recombinant ELI025).

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derived 686.9 peak, showed that the corresponding peptides had nearly-identical spectra (Fig. 6B), and matched the same peptide sequence (KNQQCLALLDAVKA) predicted for ELI025. Similarly, MASCOT analyses of MS/MS data of the 10- and 15-kDa SDS-PAGE band-derived 853.9 peak, revealed that the corresponding peptides had nearly-identical spectra (data not shown), and matched another peptide sequence (KATNPSDCVLVFNDVRL) predicted for ELI025.

Three serum samples each from pythiosis patients and normal individuals (control) were used as primary antibodies in Western blot to detect rELI025 or nELI025 in SABH and CFA. All control sera did not detect any proteins in SABH and CFA (data not shown). While pythiosis sera detected relatively-high molecular weight proteins of SABHs and CFAs (> 30 kDa), they failed to detect many lower molecular weight proteins, including the 10- and 15-kDa (representing nELI025). The patient sera also failed to react with the 12.4-kDa rELI025 band (Fig. 4C).

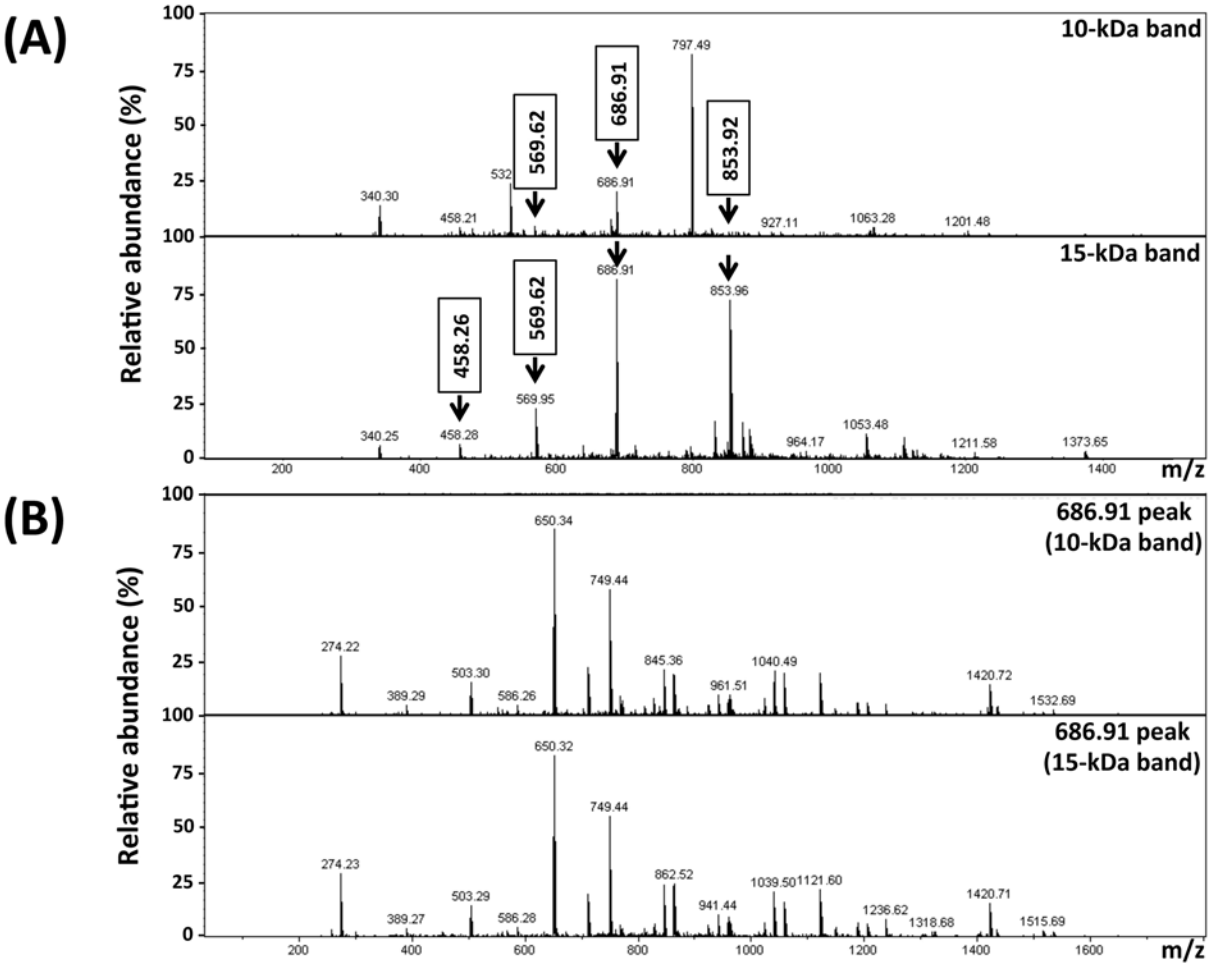

Fig 6. Mass spectrometric analyses of ELI025 by LC-MS/MS. (**A**) MS spectra of the 10- and 15-kDa SDS-PAGE band-derived proteins. The arrows indicate peptides with the mass-to-charge ratio (m/z), including 458.3, 569.6, 686.9 and 853.9, that match the ELI025 protein. Peptide sequences corresponding to the ELI025-matched peaks are shown in Table 2; (**B**) MS/MS spectra of the 686.91 peaks from the 10- and 15-kDa SDS-PAGE band-derived proteins.

doi:10.1371/journal.pone.0118547.g006

Table 2. Mass spectrometric analyses of the 10- and 15-kDa SDS-PAGE band-derived proteins showing mass-to-charge ratio (m/z), average mass of peptide (Mr; calculated by MASCOT software), peptide sequences (identified by MASCOT software), BLAST search result (against ~15,000 genome-derived predicted proteins of P. insidiosum), and amino acid position of identified peptides.

SDS-PAGE band	m/z	M_r	Peptide sequence	BLAST search result	Amino acid position
10-kDa	569.62	1705.81	KATNPSDCVLVFNDVRL	ELI025	84–100
10-kDa	686.91	1371.72	KNQQCLALLDAVKA	ELI025	72–85
10-kDa	853.92	1705.81	KATNPSDCVLVFNDVRL	ELI025	84–100
15-kDa	458.26	1371.72	KNQQCLALLDAVKA	ELI025	72–85
15-kDa	569.62	1705.81	KATNPSDCVLVFNDVRL	ELI025	84–100
15-kDa	686.91	1371.72	KNQQCLALLDAVKA	ELI025	72–85
15-kDa	853.92	1705.81	KATNPSDCVLVFNDVRL	ELI025	84–100

doi:10.1371/journal.pone.0118547.t002

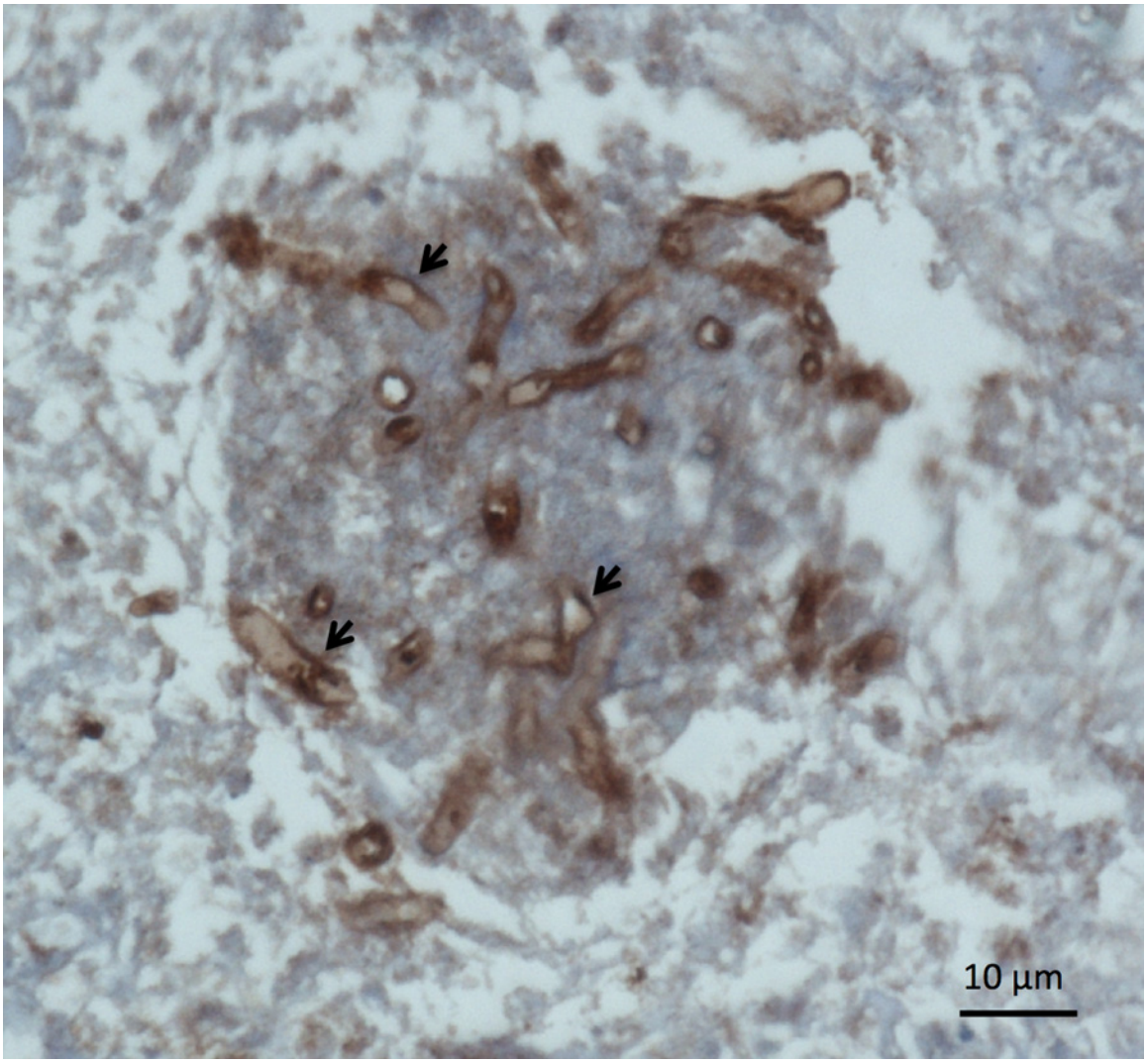

Fig 7. Cellular location of ELI025. Infected arterial tissue from a pythiosis patient was sequentially stained with rabbit anti-rELI025 serum, as the primary antibody, and then mouse anti-rabbit IgG antibody conjugated with horseradish-peroxidase, as the secondary antibody (see Materials and Methods). Images of the hyphae and location of ELI025 (indicated by arrows) were captured with a bright-field microscope. The scale bar represents 10 µm.

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An immunohistochemical staining assay, using the rabbit anti-rELI025 serum, was used to target cellular localization of the *P. insidiosum* nELI025 in an infected arterial tissue. nELI025 markedly localized at the cell surface and surrounding areas (Fig. 7). No signal was detected with the rabbit pre-immune serum.

Discussion

The elicitin domain of ELI025 was predicted to contain three disulfide bonds (Fig. 1B), which is a crucial characteristic of elicitin [34]. The *E. coli* strain rosetta-gami2 (DE3) was used to express ELI025 based on its reported facility in proper disulfide bond formation. The rabbit anti-rELI025 antibody, detected two proteins in CFA (10- and 15-kDa), but not in SABH (Fig. 4B). The rabbit anti-rELI025 serum, pre-absorbed with rELI025, failed to effectively detect any proteins in CFA, indicating that the rabbit anti-rELI025 antibodies were specific to ELI025. The 10- and 15-kDa bands could be different proteins (i.e., other elicitins) or different isoforms of

the same protein (ELI025 contains several predicted glycosylation linkages). Deglycosylation of CFA proteins indicated that the 10-kDa band represents nELI025 without glycosylation, while the 15-kDa band represents nELI025 with predominant N-linked glycosylation (Fig. 5). Thus, nELI025 is a secreted glycoprotein, with two isoforms. The slightly-larger size of rELI025 (12.4 kDa) compared to the non-glycosylated nELI025 (10 kDa) is expected based on its expression in *E. coli* as a fusion with Thrombin and a His tag (Fig. 1A).

Mass spectrometric analyses were used to confirm the identity of ELI025 in the 10- and 15-kDa bands of SDS-PAGE gel (Fig. 4A). The sequences, determined by MS and MS/MS analyses, of the peptide mass 686.9 (KNQQCLALLDAVKA) and 853.9 (KATNPSDCVLVFNDVRL) of either the 10- or 15-kDa SDS-PAGE bands matched perfectly with the ELI025 predicted protein sequence. However, no peptide masses matching ELI025 were detected in the 10- and 15-kDa Western blot bands. This may result from the Western blot bands being contaminated with blocking reagent, primary antibody, secondary antibody, enzyme, and substrate, which may compromise detection sensitivity. As an alternative method to determine the identity of the 10- and 15-kDa bands in PVDF membrane, we used rabbit anti-ELI025 antibodies against rELI025 in Western blot analysis. The rabbit anti-rELI025 antibodies reacted only with the 10- and 15-kDa bands (Fig. 4B), suggesting that the protein detected in the Western blots is ELI025.

Until recently, there were few genetic and molecular studies done in *P. insidiosum*, and to date, there is no transformation system for introducing foreign or modified *P. insidiosum* genes into the organism. In other oomycetes, transformations systems have been developed, and some of these depend on the *hsp70* and *ham34* promoters from the oomycete *Bremia lactucae* for transgene expression [78–81]. Since the upstream region of *ELI025* has conserved sequence found in the core promoter elements of many oomycete genes including *hsp70* and *ham34* (Fig. 2), it may be possible to use already-developed transformation vectors such as pTH210, pHAMT34H, pHAMT35N/SK, and pHAMT35G [79], which utilize the *hsp70* and *ham34* promoters, for developing transformations systems in *P. insidiosum*. In addition, since *ELI025* is highly expressed, its upstream region could be used as a driving promoter for DNA transformation in *P. insidiosum*.

Elicitins form a unique group of proteins that have been found previously in two oomycete genera (*Phytophthora* spp. and some *Pythium* spp.), but not in fungi or bacteria [22,82,83]. In this study, we performed a similarity search of elicitin domain-containing proteins in the publicly-available genome, transcriptome, and proteome databases of various oomycetes (Table 1). In addition to *Phytophthora* and *Pythium* species, elicitin homologs were also found in oomycete genera *Pseudoperonospora*, *Hyaloperonospora*, and *Albugo* (Table 1). As expected, phylogenetic analysis grouped the top BLAST hit elicitins of these oomycetes according to their genera based on previous classifications: *Phytophthora* species in Gr1 (with an exception for *P. vexans*), *Pythium* species in Gr2 and Gr3 (with an exception for *H. arabidopsis*), *Albugo* species in Gr4, and *Pseudoperonospora* species in Gr5 (Fig. 3B). The conserved homology of elicitins among the closely related species also extended to both their core promoter sequences (Fig. 2) and their signal sequences (Fig. 3A). It should be noted that elicitins found thus far are in the more closely-related subgroups Pythiales, Peronosporales, and Albuginales. In contrast, no elicitin homologs were detected in *Aphanomyces* and *Saprolegnia* species, which belong to Saprolegnales, a more distantly-related oomycete lineage. This finding suggests that the origin and expansion of elicitins occurred after splitting off the oomycetes from its ancient progenitor and between the Saprolegnales lineage, and the ancestor lineage of the Pythiales, Peronosporales, and Albuginales.

Based on an extensive genome search (Table 1), elicitins are found in many oomycetes, but absent in all non-oomycete organisms, such as, fungi. Thus, elicitins are a signature character

of the oomycetes. Among oomycetes, *P. insidiosum* is a notorious human pathogen. It shares microscopic features with some pathogenic fungi (such as, *Aspergillus* species, *Fusarium* species, and Zygomycetes). This can lead to misdiagnosis of pythiosis as a fungal infection [46,84], and results in delayed and improper treatment of patients. Because of the uniqueness of the elicitins to *P. insidiosum* among human pathogens, detection of *ELI025* or its gene product could aid in the development of more specific diagnostic tests for pythiosis, such as using the anti-rELI025 antibodies to detect *P. insidiosum* in infected tissue.

The detection of ELI025 in CFA, together with the predicted amino acid sequence harboring a signal peptide, indicate that ELI025 is a secreted protein. Additionally, the immunohistochemical staining assay of the infected tissue from a pythiosis patient showed localization of ELI025 at *P. insidiosum*'s cell surface and surrounding areas (Fig. 7). This evidence suggest that, in *P. insidiosum*, ELI025 is expressed and secreted, both during *in vitro* growth and during infection of host tissue. Elicitins, secreted by the plant-pathogenic oomycetes, are beneficial to the pathogens by effecting host response and triggering programed cell death [23,24]. The role of elicitin secreted by *P. insidiosum* in humans is unknown. *Pythium* species are thought to be sterol auxotrophic microorganisms [22,32,33]. Like the elicitins from the plant-pathogenic oomycetes, *P. insidiosum* ELI025 has been predicted to contain a hydrophobic cavity that can bind a sterol molecule, implying that it can function as a sterol-carrying protein [31,34,85–88]. Western blot assays showed that the small proteins ($<$ 30 kDa) in CFA, including nELI025, were not recognized by sera from patients with pythiosis (Fig. 4C). Poor immunogenicity could prevent the elimination of ELI025 by host antibody responses, and therefore, it could allow ELI025 to act in sterol acquisition inside host tissue.

In conclusion, ELI025 has been successfully cloned and expressed in *E. coli*. Genetic, biochemical, and immunological characterization showed that ELI025 is a small glycoprotein, abundantly secreted by *P. insidiosum*. ELI025 had two isoforms (glycosylated and non-glycosylated form), and was not recognized by host antibodies. The upstream region of *ELI025* shared core promoter elements with the promoters of other oomycete genes. Among human fungal and oomycete pathogens, ELI025 is unique to *P. insidiosum*, and therefore, it is a potential target for development of more specific diagnostic tests. Characterization of ELI025 provided a new insight into the biology and pathogenesis of the understudied microorganism, *P. insidiosum*, and it could lead to a discovery of a new strategy for infection control.

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Author Contributions

Conceived and designed the experiments: T. Lerksuthirat TK. Performed the experiments: T. Lerksuthirat T. Lohnoo WY RI TR RK NP. Analyzed the data: T. Lerksuthirat SR TDS TK. Contributed reagents/materials/analysis tools: T. Lerksuthirat T. Lohnoo WY RI TR SR TK. Wrote the paper: T. Lerksuthirat SR TDS TK.

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Geographic variation in the elicitin-like glycoprotein, ELI025, of *Pythium insidiosum* isolated from human and animal subjects

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ABSTRACT

Oomycetes are fungus-like in appearance, but form a distinct clade within the eukaryotes. While most pathogenic oomycetes infect plants, the understudied oomycete *Pythium insidiosum* infects humans and animals, and causes a life-threatening infectious disease, called pythiosis. Phylogenetic analyses divide *P. insidiosum* into 3 groups, according to geographic origins: Clade-I (Americas), Clade-II (Asia and Australia), and Clade-III (Thailand). Surgical removal of the infected organ is the inevitable treatment for patients with pythiosis, but it is often too late or unsuccessful, and many patients die from advanced infection. Understanding *P. insidiosum*'s basic biology could lead to improved infection control. Elicitins, a unique group of proteins found only in oomycetes, are involved in sterol acquisition and stimulation of host responses. Recently, we identified glycosylated and non-glycosylated forms of the elicitin-like protein, ELI025, which is secreted by *P. insidiosum*, and detected during *P. insidiosum* infection. In this study, we investigated geographic variation of ELI025 in 24 *P. insidiosum* strains isolated from humans, animals, and the environment. Genotypes of *ELI025*, based on 2 sets of PCR primers, correlated well with rDNA-based phylogenetic grouping. Unlike strains in Clade-I and -II, Clade-III strains secreted no glycosylated ELI025. Sera from 17 pythiosis patients yielded a broad range of antibody responses against ELI025, and ~30% lacked reactivity against the protein. Selective production or secretion of glycosylated ELI025 by different *P. insidiosum* strains might contribute to the variable host antibody responses. In conclusion, ELI025 was secreted by all *P. insidiosum* strains isolated from different hosts and geographic origins, but the protein had different biochemical, and immunological characteristics. These finding contribute to the better understanding of the biology and evolution of *P. insidiosum*, and could lead to appropriate clinical application of the ELI025 protein for diagnosis or treatment of pythiosis.

1. Introduction

Oomycetes are fungus-like microorganisms that genetically, biochemically, and physiologically differ from other eukaryotes, including fungi and parasites (Beakes et al., 2012; Kamoun, 2003; Mendoza et al., 1993). While most pathogenic oomycetes infect plants and some infect animals, the understudied oomycete *Pythium insidiosum* is capable of infecting humans and other animals, and causes the life threatening infectious disease, called pythiosis (De Cock et al., 1987; Kamoun, 2003; Mendoza and Vilela, 2013). *P. insidiosum* inhabits tropical and subtropical areas of the world (Gaastra et al., 2010; Mendoza and Vilela, 2013). Phylogenetic analyses divide *P. insidiosum* from different geographic origins into 3 groups: Clade-I (strains from Americas), Clade-II (strains from Asia and Australia continents), and Clade-III (strains from mostly Thailand) (Chaiprasert et al., 2010; Schurko et al., 2003). Almost all cases of pythiosis in humans have been reported from Thailand, whereas cases of pythiosis in animals have been found worldwide (Gaastra et al., 2010; Krajaejun et al., 2006; Mendoza, 2008; Mendoza and Vilela, 2009). No effective antimicrobial drug is currently available for treatment of *P. insidiosum* infection (Krajaejun et al., 2006; Permpalung et al., 2015). Extensive surgical removal of the infected organ (eye or leg) is an only treatment option for controlling the disease. Many patients with advanced infection die. Prompt and effective treatment could reduce the morbidity and mortality rates of pythiosis. Better

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understanding the basic biology of *P. insidiosum* is essential for developing effective infection controls.

Elicitins form a unique group of proteins that are present only in oomycetes (especially *Pythium* and *Phytophthora* species), but absent in all other organisms (Gaulin et al., 2010; Jiang et al., 2006; Madoui et al., 2009). In plant-pathogenic oomycetes, elicitins are involved in multiple biological and pathological processes, such as, acquisition of exogenous sterols (oomycetes are sterol auxotrophs), stimulation of host innate immunity (elicitins can function as a pathogen-associated molecular pattern molecules), and induction of host tissue necrosis (Boissy et al., 1996, 1999; Kamoun et al., 1998; Mikes et al., 1998, 1997; Nurnberger et al., 2004; Osman et al., 2001; Qutob et al., 2003; Yu, 1995). Recently, we have identified and characterized the elicitin-like glycoprotein, ELI025, from *P. insidiosum* (Lerksuthirat et al., 2015). *P. insidiosum* secrets large amount of glycosylated (15 kDa in size) and non-glycosylated (10 kDa) ELI025. ELI025 is abundant at the site of *P. insidiosum* infection. Its expression is up-regulated, upon exposure to body temperature. Failed recognition by host antibodies could prevent elimination of ELI025, and lead to ELI025 persistence during infection.

In this study, we characterize and compare the ELI025 protein secreted by 24 different strains of *P. insidiosum* from various isolation sources (i.e., humans, animals, and environment) and phylogenetic origins (i.e., Clade-I, -II, and -III). The information obtained could lead to appropriate clinical applications of this unique protein for the diagnosis and/or treatment of pythiosis.

2. Materials and methods

2.1. Strains, growth condition, and antigen preparation

Twenty-four strains of *P. insidiosum* isolated from patients with pythiosis ($n = 14$), animals with pythiosis ($n = 8$), environment ($n = 1$), and unknown source of isolation ($n = 1$) were used in this study (Table 1). The organisms were maintained on Sabouraud dextrose (SD) agar at room temperature, and subcultured once a month, until use. To prepare hyphal material for crude protein extraction, several small pieces of SD agar with actively-growing *P. insidiosum* colony were transferred to a flask with 100 ml SD broth, and incubated with shaking at 37 °C for 7 days. After thimerosal [0.02% (wt/vol); Sigma] was added to the culture, the hyphae were filtered through a Durapore membrane filter (0.22-μm pore size; Millipore) and used for genomic DNA extraction (see below). Cell-free broth cultures, from each of the *P. insidiosum* strains Pi05, Pi07, Pi09, Pi11, Pi20, Pi35, Pi44, Pi45 and Pi49 (Table 1), were used to prepare culture filtrate antigens or CFA (representing secreted proteins), using the protocol described by Krajaejun et al. (2002). Protein concentration was determined using a Biorad Bradford assay kit (Bradford, 1976). CFA was stored at −20 °C until use.

2.2. Serum samples

Seventeen serum samples from Thai patients with pythiosis were obtained for immunological characterization of the ELI025 protein. These patients were diagnosed by either culture identification (Chaiprasert et al., 1990) or detection of anti-*P. insidiosum* antibody (Imwidthaya and Srimuang, 1989; Jindayok et al., 2009; Krajaejun et al., 2009, 2002; Vanittanakom et al., 2004). Control sera were obtained from healthy blood donors (Blood Bank Division, Department of Pathology, Ramathibodi Hospital; $n = 8$), thalassemic patients ($n = 4$), patients with positive antinuclear antibody ($n = 1$) or rheumatoid factor ($n = 1$), and patients with other infectious diseases (two each of aspergillosis and mucormycosis, and one each of histoplasmosis, candidiasis, cryptococcosis, human immunodeficiency virus infection, hepatitis B virus infection, and syphilis). All sera were kept at −20 °C until use.

2.3. Genomic DNA extraction

Genomic DNA (gDNA) was extracted from *P. insidiosum*, using the salt-extract protocol described by Lohnoo et al. (2014). Briefly, ~500 mg of hyphal mat was transferred to a 2-ml screw-cap tube, containing glass beads (diameter, 710–1180 μm;

Table 1
Twenty-four *P. insidiosum* strains used in this study, and corresponding information, including strain ID, reference number, source of isolation, phylogenetic clade, presence (+) or absence (−) of PCR product amplified by the primer Pair#1 and Pair#2, and nucleotide accession number for *ELI025* and rDNA sequences.

Strain ID	Reference number	Source of isolation (country)	rDNA-based phylogenetic clade	PCR product		Accession number	
				Pair#1	Pair#2	ELI025	rDNA
Pi02	CBS579.85	Equine (Costa Rica)	Clade-I	+	–	–	AB971176
Pi03	CBS577.85	Equine (Costa Rica)	Clade-I	+	–	–	AB971177
Pi04	CBS576.85	Equine (Costa Rica)	Clade-I	+	–	–	AB898106
Pi05	CBS575.85	Equine (Costa Rica)	Clade-I	+	–	AB971194	AB971178
Pi06	CBS574.85	Equine (Costa Rica)	Clade-I	+	–	–	AB971179
Pi07	CBS573.85	Equine (Costa Rica)	Clade-I	+	–	AB971195	AB971180
Pi08	CBS580.85	Equine (Costa Rica)	Clade-I	+	–	–	AB898107
Pi09	CBS101555	Equine (Brazil)	Clade-I	+	–	AB971196	AB971181
Pi11	BL	Human/artery (Thailand)	Clade-II	+	+	AB898692	AB898109
Pi15	SIMI8727	Human/artery (Thailand)	Clade-II	+	+	–	AB898111
Pi16	CBS119452	Human/artery (Thailand)	Clade-II	+	+	–	AB971182
Pi20	CBS119455	Human/eye (Thailand)	Clade-II	+	+	AB898694	AB971183
Pi23	MCC10	Human/disseminated (Thailand)	Clade-II	+	+	–	AB898115
Pi26	SIMI4523–45	Human/eye (Thailand)	Clade-II	+	+	–	AB898117
Pi35	Pi-S	Human/artery (Thailand)	Clade-II	+	+	FX528334	AB898124
Pi42	CR02	Environment (Thailand)	Clade-II	+	+	–	AB971184
Pi44	CBS119454	Human/disseminated (Thailand)	Clade-III	–	–	–	AB971185
Pi45	MCC13	Human/skin (Thailand)	Clade-III	–	–	–	AB971186
Pi46	SIMI3306–44	Human/eye (Thailand)	Clade-III	–	–	–	AB971187
Pi47	SIMI2921–45	Human/eye (Thailand)	Clade-III	–	–	–	AB971188
Pi48	SIMI4763	Human/skin (Thailand)	Clade-III	–	–	–	AB971189
Pi49	SIMI7695–48	Human/artery (Thailand)	Clade-III	+	–	AB898695	AB898127
Pi50	ATCC90586	Human/skin (USA)	Clade-III	–	–	–	AB971190
Pi51	Pi51	Unknown/(Thailand)	Clade-III	–	–	–	AB898128

Sigma) and 400 μl salt homogenizing buffer [0.4 M NaCl, 10 mM Tris–HCl (pH 8.0), 2 mM EDTA (pH 8.0)], and homogenized using a Qiagen TissueLyzer MM301 machine (setting: 30 Hz for 2 min). After SDS (final concentration, 2%) and proteinase K (final concentration, 400 μg/ml) were added, the cell lysate was incubated at 55 °C for an hour, 300 μl 6 M NaCl was added, and the solution was vigorously vortexed for 30 s, and centrifuged (10,000×*g*) at room temperature for 30 min. To precipitate the gDNA, the resulting supernatant was mixed with an equal volume of isopropanol and held at −20 °C for 1 h. The pellet was collected by centrifugation, washed with 70% ethanol, air dried, and resuspended in 100 μl TE buffer (pH 8.0). The extracted gDNA was stored at −20 °C until use.

2.4. SDS–PAGE and Western blot analysis

CFA proteins were separated by SDS–PAGE (4% stacking and 12% separating gels) using a Biorad Mini-PROTEAN II apparatus (setting: 150 V for 65 min). The SDS–PAGE gel was stained with Coomassie blue R-250. Pre-stained broad range protein markers (Biorad) were used to estimate molecular weights of the separated proteins. Proteins were blotted onto a PVDF membrane (Merck Millipore), using a Biorad Mini Trans-Blot cell apparatus (setting: 100 V for 60 min). The blotted membrane was blocked with 5% skim milk (Sigma) in TBS [150 mM NaCl, 10 mM Tris–Cl pH 7.5)], and washed 3 times with TTBS [500 mM NaCl, 20 mM Tris–Cl (pH 7.5), 0.05% (v/v) Tween-20]. The membrane was incubated with the rabbit anti-ELI025 antibody (1:2000 in the blocking buffer) (Lerksuthirat et al., 2015) for 2 h at room temperature, and washed 3 times with TTBS. Goat anti-rabbit IgG, conjugated with alkaline phosphatase (Southern Biotech; 1:5000 in the blocking buffer), was added to the membrane, incubated for 2 h at room temperature, and washed 3 times with TTBS. Western blot signals were developed using BCIP and NBT.

2.5. ELISA

A 96-well polystyrene plate (Corning) was coated (100 μl/well) overnight at 4 °C with either 5 μg/ml of recombinant ELI025 [rELI025; generated by Lerksuthirat et al. (2015)] in 0.1 M sodium phosphate buffer (pH 7.4) or 5 μg/ml of CFA [prepared from the *P. insidiosum* strain Pi35] in 0.1 M carbonate buffer (pH 9.6) and 1.5% NaCl. Unbound proteins were removed by washing 4 times with TPBS pH 7.4 (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.76 mM KH_2PO_4 and 0.05% Tween-20). Each well was blocked with 250 μl of 0.5% (w/v) BSA (Merck) in 0.1 M sodium phosphate buffer (pH 7.4) at 37 °C for 1 h, and washed 4 times with TPBS. Serum samples diluted in PBS pH 7.4 (1:800 for ELI025; 1:1600 for CFA) were added to each well (100 μl/well), and incubated at 37 °C for 1 h. The plate was washed 4 times with TPBS. The goat anti-human IgG conjugated with peroxidase (Jackson Immuno Research; 1:100,000 in PBS pH 7.4) was added to each well (100 μl/well), and incubated at 37 °C for 1 h. After the plate was washed 4 times with TPBS (pH 7.4), color signal was developed using an ELISA substrate kit (Biorad). The reaction was stopped by adding 0.3 N sulfuric acid. ELISA signal was measured at OD_{450} using an Infinite 200Pro microplate reader (Tecan). ELISA signals were analyzed by the GraphPad Prism program version 5 (GraphPad Software, USA). Statistical difference between two sets of ELISA signals was determined by two-tailed unpaired *t*-test.

2.6. Polymerase chain reaction

All PCR amplifications were carried out in a Mastercycler Nexus thermal cycler (Eppendorf). Two independent pairs of primers were used to amplify portions of the ELI025 sequence: Pair#1 [ELI025_F1 (5′-TACAACGAGACCAAGCCGTG-3′) and ELI025_R1 (5′-GGCCTTGCAGCTCGTCTC-3′)]; and Pair#2 [ELI025-full-F1 (5′-C ACGCGGTGTTCGTTCCATG-3′) and ELI025-full-R1 (5′-GCGTCAAGA TGAGAAACGAGG-3′)]. The primer Pair#1 (0.5 μM each primer) were included in a 20-μl PCR reaction containing 100 ng gDNA and the KAPA Tag PCR kit reagent (Kapa Biosystems, USA) (PCR condition: 35 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 40 s). The primer Pair#2 (0.5 μM each primer) were included in a 50-μl PCR reaction containing 100 ng gDNA, 0.02 U/μl DNA polymerase (Phusion), 1× Phusion buffer, and 200 μM dNTPs (PCR condition: 98 °C for 30 s, 35 cycles of 98 °C for 10 s and 72 °C for 40 s, and 72 °C for 10 min). The primer ITS1 (5′-TCCGTAGGTGAACCTG CGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White et al., 1990) were used to amplified rDNA sequences in a 20-μl reaction, containing 100 ng gDNA, 0.025 U/μl Taq DNA Polymerase (Fermentas), 1× KCl buffer, and 200 μM dNTPs (PCR condition: 95 °C for 6 min, 35 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min, and 72 °C for 10 min). All PCR products were purified using a NucleoSpin Gel and PCR Clean-up kit (Macherey–Nagel) and assessed by 1% agarose gel electrophoresis.

2.7. DNA sequencing and nucleotide sequence accession numbers

The *ELI025* and rDNA PCR products were sequenced using the corresponding primers (Pair#1, Pair#2, or ITS1/4), and an ABI PRISM BigDye™ terminator cycle sequencing ready reaction kit, version 3.1 (Applied Biosystems, USA). Automated sequencing was performed in an ABI 3100 Genetic Analyzer, using the Applied Biosystems Sequencing software (Applied Biosystems, USA). All *ELI025* and rDNA sequences obtained in this study have been submitted to DNA data bank of Japan (DDBJ), under the accession numbers shown in Table 1.

2.8. Bioinformatic and phylogenetic analyses

ELI025 coding and deduced amino acid sequences were aligned and analyzed using ClustalX (Larkin et al., 2007) and GeneDoc (Nicholas et al., 1997). Protein domains were identified using the NCBI conserve domain search (Marchler-Bauer et al., 2015). O- and N-linked glycosylation sites were predicted online at the NetNGlyc 1.0 and NetOGlyc 4.0 servers (www.cbs.dtu.dk/services/) (Julenius et al., 2005).

rDNA sequences from 24 strains of *P. insidiosum* (Table 1) were subjected to phylogenetic tree construction, using the online program at http://www.phylogeny.fr/ (Dereeper et al., 2008). All sequences were aligned by MUSCLE (Edgar, 2004), phylogenetically analyzed by the Neighbor-Joining algorithm with 1000 bootstraps (Saitou and Nei, 1987) and the Kimura 2 parameters substitution model (Kimura, 1980). Trees were constructed using TreeDyn (Chevenet et al., 2006). rDNA sequences from *Pythium aphanidermatum* (Accession number: AY151180), *Pythium deliense* (AY151181), *Pythium grandisporangium* (AY151182), and *Lagenidium giganteum* (AY151183) were included as outgroups.

3. Results

3.1. Phylogenetic analysis of P. insidiosum

rDNA-based Neighbor-Joining phylogenetic analysis classified 24 *P. insidiosum* strains into 3 clades: Clade-I, -II, and -III (Table 1 and Fig. 1). Clade-I contained strains from the Americas [Costa Rica ($n = 7$) and Brazil ($n = 1$)]. All 15 Thai strains were grouped in Clade-II ($n = 8$) and -III ($n = 7$). One strain isolated from a pythiosis patient living in USA was placed in Clade-III. Clade-II was more closely related to Clade-I than to Clade-III. All *P. insidiosum* strains

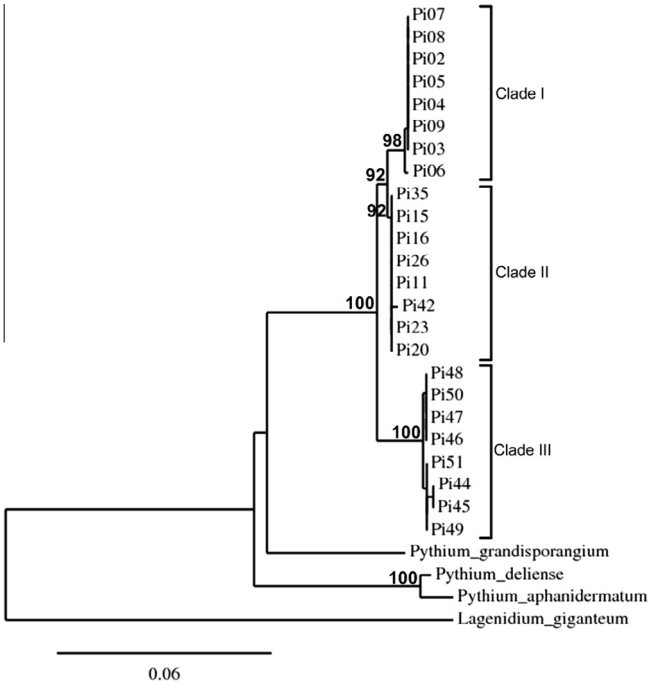

Fig. 1. Phylogenetic analysis of *P. insidiosum*: rDNA sequences from 24 strains of *P. insidiosum* (Table 1) and 4 outgroup oomycetes, including *Pythium aphanidermatum* (accession number, AY151180), *Pythium deliense* (AY151181), *Pythium grandisporangium* (AY151182), and *Lagenidium giganteum* (AY151183), are used for Neighbor-Joining based phylogenetic analysis. The reliability of the inferred trees was tested using 1000 bootstraps. The branch support values of at least 70% are shown. The scale bar at the bottom refers to the rate of nucleic acid substitution.

formed a group that is distinct from the outgroup species (i.e., other *Pythium* species and *L. giganteum*; Fig. 1).

3.2. Genetic and biochemical variations of ELI025

Two sets of primers (Pair#1: ELI025_F1 and ELI025_R1; Pair#2: ELI025-full-F1 and ELI025-full-R1) were designed to amplify *ELI025* coding and flanking sequences from gDNAs of 24 *P. insidiosum* strains (Table 1 and Fig. 2A). Pair#1 amplified a ~280 bp amplicon from all strains in Clade-I and -II, and one strain (Pi49) in Clade-III (Fig. 2B). Pair#1 produced multiple faint bands in most of Clade-III strains (Fig. 2B). Pair#1-derived PCR products of Clade-II strains were more prominent than that of Clade-I and -III strains. Pair#2 amplified an intense band (with slight size variation among the strains, ranging from ~1080 bp to ~1120 bp) from all Clade-II strains, but did not amplify any PCR product from other strains (Fig. 2B). The universal fungal rDNA primer, ITS1/4 (White et al., 1990), amplified the expected PCR product (~930 bp) from all strains (Fig. 2B).

Pair#1-derived PCR products (*ELI025*-coding region; Fig. 2A and B), amplified from the representative strains of Clade-I (Pi05, Pi07, and Pi09), Clade-II (Pi11, Pi20, and Pi35), and Clade-III (Pi49), were sequenced and compared. Alignment of all *ELI025*-coding sequences (273-bp long) showed 93–99% identity, and 18 sites of single-nucleotide polymorphism (Fig. 3A). Alignment of the corresponding translated 91-amino acid sequences revealed 97–100% identity between the strains, with three positions of amino acid polymorphisms: position 25 [Glutamate (E) for Clade-I strains; Glutamine (Q) for Clade-II and -III strains], position 29 [Lysine (K) for Clade-I strains; Threonine (T) for Clade-II and -III strains], and position 49 [Valine (V) for Clade-I strains; Isoleucine (I) for Clade-II and -III strains] (Fig. 3B). Conserved cysteines were found in all strains and are predicted to form three internal disulfide bonds: cysteine position 7 and 71, 27 and 56, and 51 and 90 (Fig. 3B, labels C1, C2 and C3). Three predicted O-linked glycosylation sites (position 29, 31 and 34) and two N-linked glycosylation sites (position 2 and 67) were present in all sequences (Fig. 3B, labels O and N).

Western blot analysis of CFA (crude extract representing *P. insidiosum* secreted proteins; Fig. 4A) was performed using rabbit anti-ELI025 antibodies. Both 15- and 10-kDa proteins [which represent glycosylated and non-glycosylated forms of ELI025, respectively (Lerksuthirat et al., 2015)] were detected in the representative strains of Clade-I (Pi05, Pi07, Pi09) and Clade-II (Pi11, Pi20, Pi35) (Fig. 4B). In contrast, only the 10-kDa protein (non-glycosylated ELI025) was detected in the representative strains of Clade-III (Pi44, Pi45, Pi49; Fig. 4B). Lower amounts of the 10- and 15-kDa proteins were detected for strain Pi09, compared to the other strains (Fig. 4B).

3.3. Immunoreactivity of ELI025 against sera from pythiosis patients

ELISA assays were performed with rELI025-coated plates and sera from pythiosis patients ($n = 17$) and controls ($n = 24$). The mean ELISA signal for pythiosis sera was 8.2-fold higher than the signal for control sera [0.882 (SD, 0.573) vs. 0.108 (SD, 0.101); $P < 0.0001$; Fig. 5B]. By comparison, ELISAs performed with

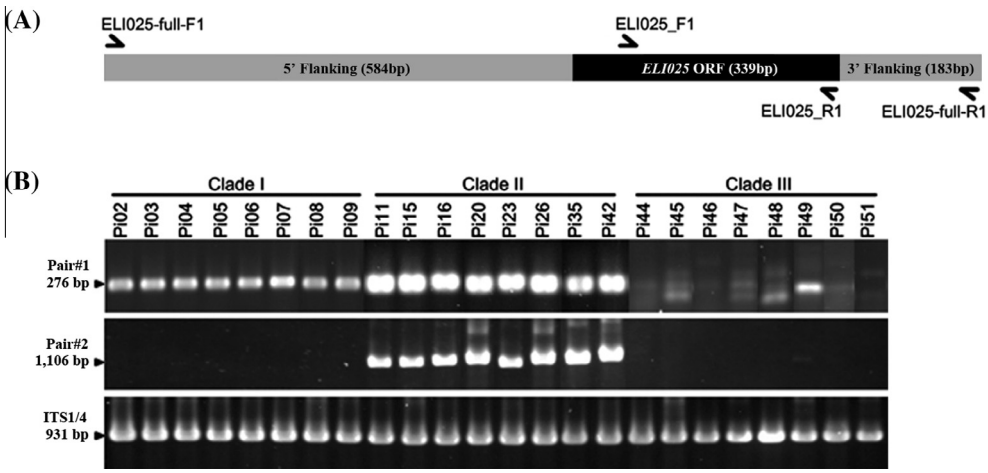

Fig. 2. PCR amplification of *ELI205* and rDNA: (A) Structure of the *ELI025* open reading frame (ORF; labeled black) and flanking (labelled gray) sequences; primer annealing sites are indicated: Pair#1 (ELI025_F1 and ELI025_R1) and Pair#2 (ELI025-full-F1 and ELI025-full-R1); (B) agarose gel electrophoresis of PCR products amplified by primer Pair#1 (*ELI025* coding sequence; 276 bp), Pair#2 (*ELI025* coding and flanking sequences; 1106 bp), and ITS1/ITS4 (rDNA sequences; 931 bp).

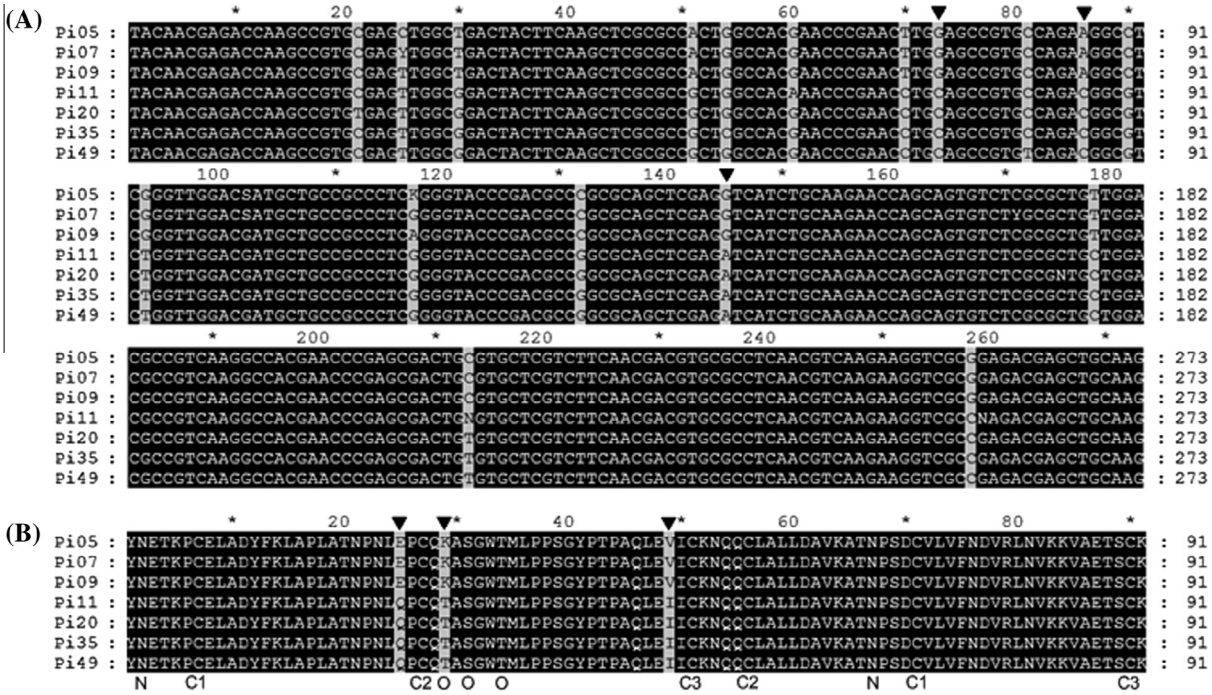

Fig. 3. Sequence analyses of ELI025: (A) alignment of the ELI025-coding sequences (273 bp) from 7 different *P. insidiosum* strains shows 18 sites (gray) of nucleotide polymorphism (arrow heads indicate nucleotide substitutions that result in amino acid changes); (B) alignment of the *ELI025*-translated amino acid sequences (91 amino acids long) demonstrates 3 amino acid changes (arrow heads), 3 predicted disulfide bonds (C1, C2, C3), and 3 predicted O-linked glycosylation sites (O), and 2 predicted N-linked glycosylation sites (N).

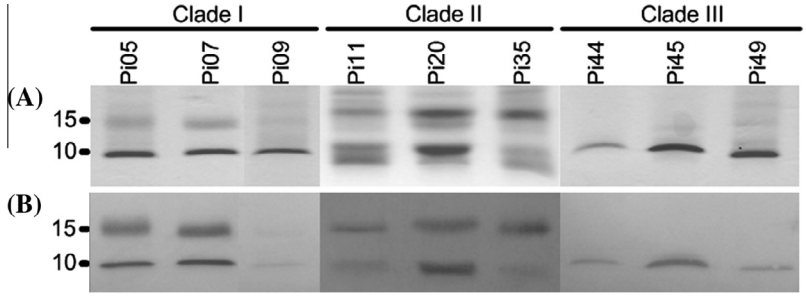

Fig. 4. SDS–PAGE and Western blot analysis of *P. insidiosum* proteins: (A) Coomassie blue stain of SDS–PAGE gel of culture filtrate antigen (CFA) prepared from the representative Clade-I, -II, and -III *P. insidiosum* strains; (B) Western blot analysis shows the separated CFAs, after probing with the rabbit anti-ELI025 antibody. Estimated protein molecular weights (10 and 15 kDa) are shown on the left.

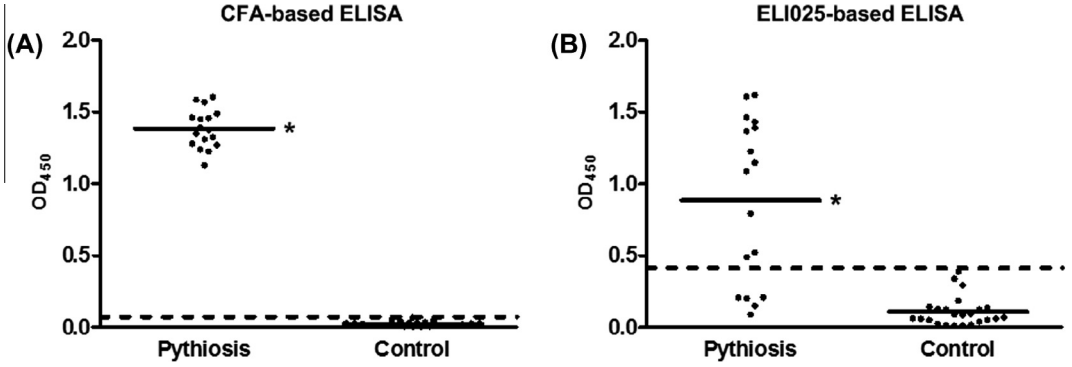

Fig. 5. Immunoreactivity of CFA and ELI025 against pythiosis and control sera: (A) CFA-based ELISA shows that CFA is recognized by pythiosis sera ($n = 17$), but not by control sera ($n = 24$); (B) ELI025-based ELISA shows that ELI025 has variable immunoreactivity against pythiosis sera, and was minimally recognized by control sera. The solid line represents mean ELISA signal (measured at OD_{450}). Dash line shows ELISA cut-off value (calculated by mean ELISA signal of control sera plus 3 SDs). Asterisk indicates significant difference ($P < 0.0001$) between mean ELISA signals of pythiosis and control sera.

CFA-coated plates had a mean ELISA signal for pythiosis patient sera which was 55.3-fold higher than that of control sera [1.382 (SD, 0.136) vs. 0.025 (SD, 0.016); $P < 0.0001$; Fig. 5A].

A cut-off value [defined as the mean ELISA signal of control sera plus 3 SDs (Krajaejun et al., 2002)] was used to differentiate a positive test reaction from controls. All control sera had ELISA signals below the CFA- and rELI025-based cut-off values, indicating both assays had 100% detection specificity (Fig. 5A and B). For the CFA-based ELISAs, all pythiosis patient sera had values above the cut-off (0.073) for a 100% detection sensitivity (Fig. 5A). By contrast, only 12 of the 17 pythiosis patient sera had rELI025-based ELISA values above the cut-off (0.410) to give 71% detection sensitivity (Fig. 5B).

4. Discussion

Based on the rDNA sequences, all 24 *P. insidiosum* strains, used in this study (Table 1), can be classified into 3 phylogenetic groups, Clade-I, -II, and -III (Fig. 1), which is consistent with previously reported phylogenetic classifications (Schurko et al., 2003; Chaiprasert et al., 2010). Fragments produced by PCR using primer Pair#1 and Pair#2, (targeting *ELI025*-coding and -flanking sequences of *P. insidiosum*; Fig. 2A), were used to characterize the *ELI025* gene of clades-I, -II, and -III. The primer Pairs#1 and #2 successfully amplified product for 17 strains and 8 strains, respectively (Fig. 2B), suggesting that the *ELI025* sequences are genetically variable, and the primer annealing sites of Pair#1 were more conserved than that of Pair#2. The patterns of positive and negative PCR reactions were clade-specific, and correlated with the rDNA sequence based phylogenetic groups: Pair#1(+)/Pair#2 (−) for Clade-I strains, (+)/(+) for Clade-II strains, and (−)/(−) for Clade-III strains [with one exception for strain Pi49: (+)/(−)] (Table 1 and Fig. 2B). Thus, to a great extent, Pair#1- and Pair#2-derived PCR pattern could be used as a simple method for genotyping *P. insidiosum*, that could abrogate the necessity for rDNA sequence analyses. For example, the (+)/(+) pattern suggests Clade-II genotype, while the (−)/(−) pattern suggests Clade-III genotype. However, if the (+)/(−) pattern is observed, rDNA sequence analysis is required to discriminate Clade-I and Clade-III genotypes. Further study using a greater number of *P. insidiosum* strains is necessary for evaluation of this genotyping method.

All PCR patterns [(+)/(+), (−)/(−), or (+)/(−)] of the *ELI025* sequence were observed in the isolates from all human patients (who had different site of infection, i.e., eye, artery, skin), while only the (+)/(−) PCR pattern was observed in the isolates from animals (Table 1 and Fig. 2B). These findings suggest that the human hosts, regardless of clinical manifestations, were susceptible to *P. insidiosum* with all *ELI025*-based genotypes, while the animal hosts were susceptible to *P. insidiosum* with the (+)/(−) genotype.

Previously, we demonstrated that three different *P. insidiosum* strains, isolated from Thai patients with pythiosis, secreted two forms of ELI025: glycosylated (15 kDa) and non-glycosylated (10 kDa) (Lerksuthirat et al., 2015). As shown here, ELI025 was also secreted by all other *P. insidiosum* strains isolated from different sources (humans, animals, and environment) and from different phylogenetic groups (Clade-I, -II, and -III) (Table 1 and Fig. 4). Nevertheless, Clade-III strains, which are phylogenetically distinct from Clade-I and -II strains (Fig. 1), lacked the glycosylated ELI025 (15-kDa band; Fig. 4). Sequence variations in the *ELI025* gene (i.e., base changes, deletions, or insertions), which are likely responsible for different amplicon patterns (Fig. 2B), may also lead to the lack of glycosylated ELI025 in Clade-III strains. To address this issue, the *ELI025*-coding sequences from the representative Clade-I, -II, and -III strains were aligned and analyzed (Fig. 3A). All ELI025 protein sequences have predicted glycosylated sites at the amino acid positions 2 and 67 (for N-link glycosylation) and 29, 30 and 34 (for O-link glycosylation) (Fig. 3B). Eighteen sites of nucleotide polymorphism were identified (Fig. 3A), but only three sites (positions 25, 29, and 49) are associated with amino acid changes: E25-K29-V49 for Clade-I strains; and Q25-T29-I49 for both Clade-II and -III strains (Fig. 3B). Strikingly, changes in the amino acid sequence of ELI025 cannot explain the lack of the glycosylated form in Clade-III strains, because the deduced amino acid sequences of ELI025 in the Clade-II and Clade-III strains are identical. Another alteration in the Clade-III strains, such as a defect in post-translational modification or glycoproteins secretion, must explain the failure to detect glycosylated ELI025.

The crude extract, CFA, comprises hundreds of *P. insidiosum* secreted proteins, including ELI025. CFA-based ELISA showed that all sera from 17 Thai patients with pythiosis, but not sera from the control group, had robust antibody responses against *P. insidiosum* (Fig. 5A). In contrast, the rELI025-based ELISA had variable responses with the same set of pythiosis sera. While ~70% of the assays had positive responses (above the cut-off), ~30% of these sera were indistinguishable from the controls (Fig. 5B). Since Clade-I strains have never been isolated in Thailand, Thai patients that provided the sera were likely infected with a strain from either Clade-II or -III (Table 1). Host immunity might recognize and respond to each particular form of ELI025 differently. Selective production or secretion of glycosylated ELI025, observed among the Clade-II and -III strains (Fig. 4), might contribute to the variable host antibody responses to ELI025 (Fig. 5B). In the plant-pathogenic oomycete *Pythium vexans*, two secreted elicitins, Vex1 and Vex2, were identified (Huet et al., 1995). The glycosylated elicitin, Vex1, exhibits more robust toxicity in the plant host, compared to the non-glycosylated elicitin, Vex2, suggesting an important role of protein glycosylation in host response and pathogenesis.

In conclusion, ELI025, secreted by *P. insidiosum* from different phylogenetic groups, had different genetic, biochemical, and immunological characteristics. Selective production or secretion of glycosylated ELI025 by different *P. insidiosum* strains might contribute to variable host antibody responses, so that ELI025-based ELISAs would not be clinically relevant. ELI025 is a unique protein present in all strains of *P insidiosum* from different hosts and geographic origins. Therefore, direct detection of this pathogen via immunohistochemical staining of ELI025 could prove to be useful for diagnosis of *P. insidiosum* infection. In addition, based on the Clade-specific patterns of PCR outcomes using primer Pairs#1 and #2, a simple PCR test could be used in the clinic for genotyping *P. insidiosum*.

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Development of an Anti-Elicitin Antibody-Based Immunohistochemical Assay for Diagnosis of Pythiosis

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Pythiosis is an emerging and life-threatening infectious disease of humans and animals living in tropical and subtropical countries and is caused by the fungus-like organism *Pythium insidiosum*. Antifungals are ineffective against this pathogen. Most patients undergo surgical removal of the infected organ, and many die from advanced infections. Early and accurate diagnosis leads to prompt management and promotes better prognosis for affected patients. Immunohistochemical assays (IHCs) have been developed using rabbit antibodies raised against *P. insidiosum* crude extract, i.e., culture filtrate antigen (CFA), for the histodiagnosis of pythiosis, but cross-reactivity with pathogenic fungi compromises the diagnostic performance of the IHC. Therefore, there is a need to improve detection specificity. Recently, the elicitin protein, ELI025, was identified in *P. insidiosum*, but it was not identified in other human pathogens, including true fungi. The ELI025-encoding gene was successfully cloned and expressed as a recombinant protein in *Escherichia coli*. This study aims to develop a new IHC using the rabbit anti-ELI025 antibody (anti-ELI) and to compare its performance with the previously reported anti-CFA-based IHC. Thirty-eight *P. insidiosum* histological sections stained positive by anti-ELI-based and anti-CFA-based IHCs indicating 100% detection sensitivity for the two assays. The anti-ELI antibody stained negative for all 49 negative-control sections indicating 100% detection specificity. In contrast, the anti-CFA antibody stained positive for one of the 49 negative controls (a slide prepared from *Fusarium*-infected tissue) indicating 98% detection specificity. In conclusion, the anti-ELI based IHC is sensitive and specific for the histodiagnosis of pythiosis and is an improvement over the anti-CFA-based assay.

Pythiosis is an emerging and life-threatening infectious disease of humans and animals (1–4). The disease has been increasingly reported worldwide, mostly in tropical and subtropical regions. The etiologic agent is the oomycete microorganism *Pythium insidiosum*, which is fungus-like in its hyphal appearance. *P. insidiosum* is recognized as a water mold and completes its life cycle in wetland areas (5, 6). *P. insidiosum* is the only oomycete that infects humans and animals (1–4, 7). Misidentification of *P. insidiosum* as a true fungus (i.e., *Aspergillus*, *Fusarium*, zygomycetes) can occur because of the shared hyphal morphology of these pathogens (8). This leads to delayed proper management and, as a result, poor prognosis in patients with pythiosis.

Direct exposure to *P. insidiosum* initiates an infection (5). The most common clinical presentations of human pythiosis are vascular pythiosis (infection of arterial tissue resulting in occlusion and aneurysm) and ocular pythiosis (infection of corneal tissue resulting in keratitis and ulcer) (3, 4). Antifungal drugs are ineffective against *P. insidiosum*. Most patients undergo surgical removal of the affected organ (i.e., arm, leg, eye), and many patients die from advanced infection. In contrast, animals with pythiosis usually present with cutaneous or gastrointestinal tract infection (1, 2). Delayed identification of the causative agent leads to fatal outcomes for patients and animals with pythiosis.

Early and accurate diagnosis is key to the prompt and effective treatment of pythiosis. The current diagnostic modalities, including culture identification (9–11), serodiagnosis (12–22), and molecular-based detection (22–27), are fraught with problems. For example, culture identification is time-consuming and often fails to grow and to identify the organism. Serodiagnostic tests (i.e., immunodiffusion, enzyme-linked immunosorbent assay [ELISA], Western blot, hemagglutination, and immunochromatographic tests) for the detection of anti-*P. insidiosum* antibodies, usually produce false-negative results from the serum of patients with ocular pythiosis. Molecular assays, based on PCR and sequence homology, require skilled personnel and sophisticated equipment, which is not readily available in the regions where pythiosis is endemic. In addition, limited yield or degradation of the extracted DNA compromises the diagnostic performance of such assays.

As alternatives, several investigators have developed immunohistochemical assays (IHCs) for the diagnosis of pythiosis. These assays are based on rabbit antiserum (as the primary antibody) and are raised against *P. insidiosum* crude extracts (i.e., culture filtrate antigen [CFA] and soluble antigen from broken hyphae [SABH]) (28, 29). IHC showed good detection sensitivity but limited detection specificity due to cross-reactivity of the assay with

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some pathogenic fungi, i.e., *Fusarium* and *Conidiobolus* species (25, 29). Therefore, specificity of the IHCs needs to be improved.

Elicitins form a group of proteins found only in a phylogenetically distinct group of microorganisms, the oomycetes, but are absent in all other microorganisms, including true fungi (30–33). Recently, we reported a number of elicitins from the *P. insidiosum* transcriptome, and one of them, ELI025, is highly expressed and appears at the pathogen cell surface (33–35). Since the elicitins are unique to *P. insidiosum* among the human pathogens, direct detection of ELI025 can aid in the development of a more specific IHC for pythiosis. In this study, we developed a new IHC using the rabbit anti-ELI025 antibody (anti-ELI) (33) for histodiagnosis of *P. insidiosum*, and we compared its performance with the established IHC, which uses the rabbit anti-CFA antibody (anti-CFA) (29).

MATERIALS AND METHODS

Paraffin-embedded histological sections. Eighty-seven paraffin-embedded samples were prepared from pure cultures of *P. insidiosum* or other fungi (referred to as "culture blocks") (Table 1) and from infected tissues (referred to as "tissue blocks") (Table 2) for the evaluation of IHC. Nineteen strains of *P. insidiosum* (reference codes CP01 to CP19 in Table 1; isolated from the environment [$n = 2$] and patients with vascular pythiosis [$n = 9$], ocular pythiosis [$n = 4$], cutaneous pythiosis [$n = 2$], and other forms of pythiosis [$n = 2$]) and 31 isolates of other fungi (reference codes CC01 to CC31 in Table 1; served as controls and included *Fusarium* spp. [$n = 8$], *Aspergillus* spp. [$n = 4$], *Acremonium* spp. [$n = 3$], *Absidia* spp. [$n = 2$], *Epidermophyton* spp. [$n = 2$], *Geotrichum* spp. [$n = 2$], *Paecilomyces* spp. [$n = 2$], and *Trichophyton* spp. [$n = 2$] and one each of *Mucor* sp., *Chrysosporium* sp., *Cladosporium* sp., *Gliocladium* sp., *Microsporium* sp., and *Scedosporium* sp.) were obtained for culture block preparation. The identity of each organism was confirmed by culture. Each organism was grown in Sabouraud dextrose broth at 37°C for up to 10 days. Merthiolate was added to the culture at the final concentration of 0.02% (wt/vol). The organism was harvested, fixed with 10% buffered formalin, and embedded in paraffin blocks at the Department of Pathology, Ramathibodi Hospital.

A total of 37 paraffin-embedded tissue blocks, prepared from the infected tissues of 19 patients with pythiosis (reference codes TP01 to TP19 in Table 2) and 18 patients with other fungal infections (reference codes TC01 to TC18 in Table 2; served as negative controls and included *Candida albicans* [$n = 4$], *Aspergillus* spp. [$n = 3$], *Aspergillus flavus* [$n = 3$], *Aspergillus fumigatus* [$n = 2$], *Fusarium* spp. [$n = 2$], *Candida* spp. [$n = 2$], *Trichosporon cutaneum* [$n = 1$], and a phaeomycotic fungus [$n = 1$]) were obtained from Ramathibodi Hospital, Siriraj Hospital, and Chulalongkorn Hospital. The identity of each organism in the infected tissues was confirmed by histological examination and culture identification.

Each tissue or culture block was cut into 4-μm slices using a microtome (Finesse 325; Thermo Scientific, USA). Paraffin-embedded sections were placed on glass slides for downstream IHC analyses.

Grocott's methenamine silver and immunohistochemical stains. Each paraffin-embedded section was analyzed with the Grocott's methenamine silver (GMS) stain, as previously described (36), and was examined under a light microscope (Eclipse Ci; Nikon, Japan). Two different IHCs for detecting *P. insidiosum* were performed using the methods described by Keeratijarut et al. (for anti-CFA-based IHC) (29) and Lerksuthirat et al. (for anti-ELI-based IHC) (33) with some modifications. Briefly, each paraffin-embedded section was treated with xylene (to deparaffinize) and with ethanol (to replace the xylene). Slides were then washed with phosphate-buffered saline (PBS; pH 7.4) and were incubated in Tris-EDTA (TE) buffer (pH 9.0) at 95°C in a water bath for 40 min. To reduce nonspecific staining from endogenous peroxidase, the sections were treated with 10% H_2O_2 in PBS for 10 min and were then washed with PBS. The section was incubated overnight with

TABLE 1 Results of the anti-CFA-based and anti-ELI-based immunohistochemical assays using culture blocks[a]

Reference code	Organism identity	IHC result[b] for:	
		Anti-CFA[c]	Anti-ELI[d]
CP01	*P. insidiosum*	+	+
CP02	*P. insidiosum*	+	+
CP03	*P. insidiosum*	+	+
CP04	*P. insidiosum*	+	+
CP05	*P. insidiosum*	+	+
CP06	*P. insidiosum*	+	+
CP07	*P. insidiosum*	+	+
CP08	*P. insidiosum*	+	+
CP09	*P. insidiosum*	+	+
CP10	*P. insidiosum*	+	+
CP11	*P. insidiosum*	+	+
CP12	*P. insidiosum*	+	+
CP13	*P. insidiosum*	+	+
CP14	*P. insidiosum*	+	+
CP15	*P. insidiosum*	+	+
CP16	*P. insidiosum*	+	+
CP17	*P. insidiosum*	+	+
CP18	*P. insidiosum*	+	+
CP19	*P. insidiosum*	+	+
CC01	*Fusarium* sp	−	−
CC02	*Fusarium* sp	−	−
CC03	*Fusarium* sp	−	−
CC04	*Fusarium* sp	−	−
CC05	*Fusarium* sp	−	−
CC06	*Fusarium* sp	−	−
CC07	*Fusarium* sp	−	−
CC08	*Fusarium* sp	−	−
CC09	*Aspergillus* sp	−	−
CC10	*Aspergillus* sp	−	−
CC11	*Aspergillus* sp	−	−
CC12	*Aspergillus* sp	−	−
CC13	*Acremonium* sp	−	−
CC14	*Acremonium* sp	−	−
CC15	*Acremonium* sp	−	−
CC16	*Absidia* sp	−	−
CC17	*Absidia* sp	−	−
CC18	*Epidermophyton* sp	−	−
CC19	*Epidermophyton* sp	−	−
CC20	*Geotrichum* sp	−	−
CC21	*Geotrichum* sp	−	−
CC22	*Paecilomyces* sp	−	−
CC23	*Paecilomyces* sp	−	−
CC24	*Trichophyton* sp	−	−
CC25	*Trichophyton* sp	−	−
CC26	*Mucor* sp	−	−
CC27	*Chrysosporium* sp	−	−
CC28	*Cladosporium* sp	−	−
CC29	*Gliocladium* sp	−	−
CC30	*Microsporum* sp	−	−
CC31	*Scedosporium* sp	−	−

[a] Paraffin-embedded blocks prepared from pure cultures of *P. insidiosum* ($n = 19$) and true fungi ($n = 31$).
[b] +, Positive stain; −, negative stain.
[c] Rabbit anti-CFA (culture filtrate antigen) serum.
[d] Rabbit anti-ELI (ELI025) serum.

TABLE 2 Results of the anti-CFA-based and anti-ELI-based immunohistochemical assays using tissue blocks[a]

Reference code	Organism identity	Organ/tissue	GMS result[b]	IHC result[b] for:	
				Anti-CFA[c]	Anti-ELI[d]
TP01	*P. insidiosum*	Cornea	+	+	+
TP02	*P. insidiosum*	Artery	+	+	+
TP03	*P. insidiosum*	Artery	+	+	+
TP04	*P. insidiosum*	Artery	+	+	+
TP05	*P. insidiosum*	Artery	+	+	+
TP06	*P. insidiosum*	Artery	+	+	+
TP07	*P. insidiosum*	Artery	+	+	+
TP08	*P. insidiosum*	Brain	+	+	+
TP09	*P. insidiosum*	Cornea	+	+	+
TP10	*P. insidiosum*	Cornea	+	+	+
TP11	*P. insidiosum*	NA[e]	+	+	+
TP12	*P. insidiosum*	NA	+	+	+
TP13	*P. insidiosum*	NA	+	+	+
TP14	*P. insidiosum*	NA	+	+	+
TP15	*P. insidiosum*	NA	+	+	+
TP16	*P. insidiosum*	NA	+	+	+
TP17	*P. insidiosum*	NA	+	+	+
TP18	*P. insidiosum*	NA	+	+	+
TP19	*P. insidiosum*	NA	+	+	+
TC01	*Aspergillus flavus*	Nasal cavity	+	−	−
TC02	*Aspergillus flavus*	Colon	+	−	−
TC03	*Aspergillus flavus*	Lung	+	−	−
TC04	*Aspergillus fumigatus*	Trachea	+	−	−
TC05	*Aspergillus fumigatus*	Air sac wall	+	−	−
TC06	*Aspergillus* sp	Sinus	+	−	−
TC07	*Aspergillus* sp	Sinus	+	−	−
TC08	*Aspergillus* sp	Nasal cavity	+	−	−
TC09	*Candida albicans*	Diaphragm	+	−	−
TC10	*Candida albicans*	Lung	+	−	−
TC11	*Candida albicans*	Lung	+	−	−
TC12	*Candida albicans*	Heart	+	−	−
TC13	*Candida* sp	NA	+	−	−
TC14	*Candida* sp	Lip	+	−	−
TC15	*Trichosporon cutaneum*	Lung	+	−	−
TC16	Phaeomycotic fungus[f]	Skin	+	−	−
TC17	*Fusarium* sp	Cornea	+	−	−
TC18	*Fusarium* sp	Skin	+	+	−

[a] Paraffin-embedded blocks prepared from infected tissues of patients with pythiosis ($n = 19$) and other mycoses ($n = 18$).
[b] +, Positive stain; −, negative stain.
[c] Rabbit anti-CFA (culture filtrate antigen) serum.
[d] Rabbit anti-ELI (ELI025) serum.
[e] NA, data not available.
[f] A pigmented fungus that causes a phaeomycotic cyst.

200 μl of rabbit preimmune, anti-ELI (made available by Lerksuthirat et al. [33]), or anti-CFA (made available by Keeratijarut et al. [29]) serum (1:16,000 in PBS) in a moisture chamber at 4°C. After washing 3 times with PBS (5 min each), the sections were incubated at room temperature for 30 min with 200 μl of undiluted mouse anti-rabbit IgG antibody conjugated with horseradish-peroxidase (Thermo Scientific, USA). After washing as described above, color was developed with 200 μl of 3,3′-diaminobenzidine tetrahydrochloride diluted (1:200) in DAB substrate (Dako, USA), which was added to each section and incubated at room temperature for 5 min. The section was counterstained with hematoxylin for 15 min and was examined under a light microscope (Eclipse Ci; Nikon, Japan). A stain section was considered positive if organisms were stained brown and negative if organisms were unstained. Assay interpretation was determined by two independent examiners. Positive or negative calls were consistently interpreted by the two examiners.

Statistical analysis. Detection sensitivity, detection specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy were calculated using Microsoft Excel 2013 software (12).

RESULTS

Analyses of the paraffin-embedded culture blocks and tissue blocks. To test the sensitivity and specificity of the IHCs developed to detect *P. insidiosum* in infected tissues, two different types of specimens were utilized. In one set, pure cultures of 19 independent isolates of *P insidiosum* and pure cultures of 31 independent isolates of true fungi were embedded in paraffin (culture blocks; see Materials and Methods and Table 1). Another set of paraffin-embedded block specimens was produced from tissues obtained from pythiosis patients or from patients with various mycoses (tissue blocks; see Materials and Methods and Table 2).

Before proceeding to the IHC, it was important to confirm that the paraffin-embedded culture block and tissue block sections harbored the organism in question. For the culture block sections, organisms were microscopically visible without GMS staining (data not shown). For the tissue-block sections, GMS was used to stain the hyphal elements black for microscopic detection of infecting organisms. In all tissue block sections, including 19 *P. insidiosum* specimens and 18 true fungi specimens, hyphal elements were visible with GMS staining (Fig. 1A and D and data not shown).

Development of an immunohistochemical assay using an anti-elicitin antibody. Early experiments were performed to establish an optimal antibody dilution for the IHC. The rabbit anti-ELI025 antibody (anti-ELI) (33) was used as the primary antibody for the immunohistochemical staining of *P. insidiosum* in tissue sections, and the optimal dilution was determined to be 1:16,000 (see Materials and Methods; data not shown). With this dilution of anti-ELI, the hyphae of *P. insidiosum* isolates were stained brown in all of the paraffin-embedded sections tested (Fig. 1C), while the IHC using rabbit preimmune serum did not stain the organism (data not shown).

Comparison of anti-CFA-based and anti-ELI-based immunohistochemical assays. The anti-CFA-based IHC, using the rabbit anti-CFA antibody (29), and the anti-ELI-based IHC were evaluated for their diagnostic performance against the same set of culture block sections (Table 1). For the two IHCs, hyphae were

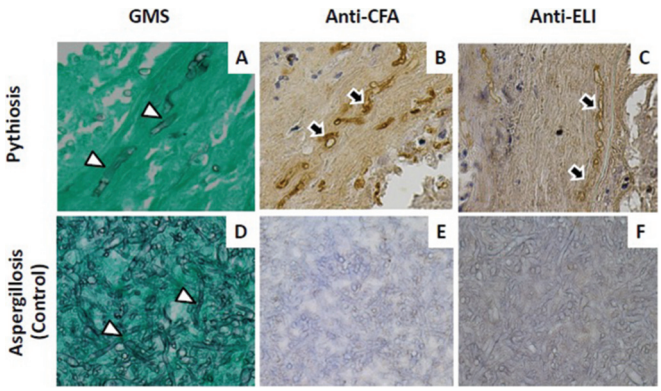

FIG 1 Immunohistochemical stains of sections from a patient with pythiosis (sample reference code TP01) (A to C) and aspergillosis (sample reference code TC01) (D to F). (A and D) Grocott's methenamine silver (GMS) stain (white arrowheads indicate organisms). (B and E) Immunohistochemical stain using the anti-culture filtrate antigen antibody (anti-CFA) as the primary antibody (arrows indicate positive stain). (C and F) Immunohistochemical stain using the anti-elicitin antibody (anti-ELI) as the primary antibody (black arrows indicate stained organisms).

TABLE 3 Diagnostic performance of the anti-CFA-based and anti-ELI-based immunohistochemical assays[a]

Sample type	Anti-CFA-based IHC[b] performance:					Anti-ELI-based IHC[c] performance:				
	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
Culture blocks[d]	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Tissue blocks[e]	100.0	94.4	95.0	100.0	97.3	100.0	100.0	100.0	100.0	100.0
Culture and tissue blocks	100.0	98.0	97.4	100.0	98.9	100.0	100.0	100.0	100.0	100.0

[a] Evaluated against the culture (*P. insidiosum*, $n = 19$; other fungi, $n = 31$) and tissue (*P. insidiosum*, $n = 19$; other fungi, $n = 18$) blocks.
[b] Immunohistochemical assay using the rabbit anti-CFA (culture filtrate antigen) serum.
[c] Immunohistochemical assay using the rabbit anti-ELI (ELI025) serum.
[d] Paraffin-embedded block prepared from pure cultures of *P. insidiosum* or other fungi.
[e] Paraffin-embedded block prepared from infected tissues of patients with pythiosis or other mycoses.

clearly detected in all 19 *P. insidiosum* culture block-derived sections, and the tests failed to stain any organisms in any of the negative-control sections (Table 1). Based on the results of all 50 culture blocks, the anti-CFA-based and anti-ELI-based IHCs attained 100% responses for detection sensitivity, detection specificity, PPV, NPV, and accuracy (Table 3).

The anti-CFA-based and anti-ELI-based IHCs were further evaluated using the tissue blocks (Table 2). The two IHCs detected organisms in all 19 *P. insidiosum* tissue block-derived sections (Table 2; Fig. 1B and C). Of the 18 control tissue-derived sections (Table 2), 17 (reference codes TC01 to TC17) were unstained by the two IHCs (Fig. 1E and F). One section (*Fusarium*-infected tissue, reference code TC18) was stained positive by the anti-CFA-based IHC, but it was stained negative by the anti-ELI-based IHC (data not shown). Based on the results of all 37 *P. insidiosum* and control tissue blocks, the anti-ELI-based IHC demonstrated 100% detection sensitivity, detection specificity, PPV, NPV, and accuracy (Table 3). While the anti-CFA-based IHC exhibited 100% detection sensitivity and NPV and 94.4% detection specificity, 95.0% PPV, and 97.3% accuracy (Table 3).

Diagnostic performance values of the two IHCs were also calculated based on the combined results of all 87 culture and tissue blocks. Detection sensitivity, detection specificity, PPV, NPV, and accuracy of the anti-ELI-based IHC were all determined to be 100% while those of the anti-CFA-based IHC were 100%, 98.0%, 97.4%, 100.0%, and 98.9%, respectively (Table 3).

DISCUSSION

IHC is a well-known technique that is routinely performed in general pathology laboratories. IHC is a useful assay for detection of *P. insidiosum* (28, 29, 37), especially when culture identification, serodiagnosis, or molecular detection is not available or fails to definitively diagnose the infecting organism in possible cases of pythiosis. Rabbit antiserum that was raised against the *P. insidiosum* crude extract (i.e., SABH and CFA, which contain various protein species) has been used as the primary tool in the development of the IHCs (28, 29, 37). These assays demonstrated high detection sensitivity. However, some investigators have observed the limited specificity of IHCs (25, 29), which is conceivably due to cross-reactivity by nonspecific antibodies in the rabbit antiserum to some fungi (i.e., *Fusarium* and *Conidiobolus*) that share microscopic features with *P. insidiosum*. Improving the diagnostic performance of IHC relied on the specificity of the anti-*P. insidiosum* antibody.

Among the human pathogens, elicitins are present only in *P. insidiosum* and not in true fungi or other microorganisms (30–35). One of the elicitins, ELI025, has been identified in *P. insidiosum* as a highly expressed protein on the cell surface (33–35). ELI025 has been successfully expressed and purified as a recombinant protein from the bacterium *Escherichia coli* (33). Due to the uniqueness of ELI025, rabbit antiserum (anti-ELI) raised against this protein (33) is expected to be specific to *P. insidiosum* and, therefore, is a good candidate for IHC development. In addition, among different *P. insidiosum* strains isolated from humans and animals living in different geographic areas across the world, ELI025 is immunologically conserved and can be detected by anti-ELI (38). In the present study, an anti-ELI-based IHC was successfully developed, and its diagnostic performance was evaluated and compared with that of the anti-CFA-based IHC (29).

Because a limited number of the paraffin-embedded tissue blocks were available ($n = 37$) (Table 2), paraffin-embedded culture blocks were prepared from 19 different *P. insidiosum* strains and 31 various fungal species, including those that share hyphal morphology with *P. insidiosum* (Table 1). Diagnostic parameters of anti-ELI-based and anti-CFA-based IHCs were analyzed (Table 1 and 2). The GMS assay (36), which stains fungal and *P. insidiosum* hyphal elements, ensured the presence of the expected organism in the sections prepared from the tissue blocks and in so doing eliminated possible false-negative results. The anti-ELI-based and anti-CFA-based IHCs correctly detected *P. insidiosum* in all culture and tissue block-derived sections, indicating that the two assays had 100% detection sensitivity (Table 1 to 3). The two IHCs were negative for the control fungi in all specimens tested, except for one sample from a patient with *Fusarium* infection (Table 2, reference code TC18) that was positive by the anti-CFA-based IHC. This indicates that the detection specificity of the anti-CFA-based IHC (98%) was slightly lower than that of the anti-ELI-based IHC (100%) within the sample set tested (Table 3). Cross-reactivity between *P. insidiosum* and *Fusarium* species was also observed by Keeratijarut et al. using the same assay (29). Since only 1 in 10 *Fusarium* specimens tested (Table 1 and 2, reference codes CC01 to CC08 and TC17 to TC18) was reactive against the anti-CFA antibody, it is likely that not all *Fusarium* species share antigens with *P. insidiosum*. As demonstrated here, the IHC using anti-ELI as the refined mono-protein-specific primary antibody retained high detection sensitivity while improving the detection specificity compared to that of the IHC using anti-CFA with multiprotein-specific primary antibodies.

In conclusion, accurate diagnosis of pythiosis is remarkably critical for proper and timely management (i.e., adequate surgical intervention), which promotes better clinical outcomes for affected patients or animals. We have successfully developed an IHC

using the anti-ELI antibodies and have improved diagnostic performance (100% sensitivity, specificity, PPV, NPV, and accuracy) for the detection of *P. insidiosum* in tissues. This is an advance in the field of *P. insidiosum* studies, where basic biological knowledge and genetic engineering technology, have facilitated the successful development of a better diagnostic assay.

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Protein A/G-based immunochromatographic test for serodiagnosis of pythiosis in human and animal subjects from Asia and Americas

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Abstract

Pythiosis is a life-threatening infectious disease of both humans and animals living in Asia, Americas, Africa, and parts of Australia and New Zealand. The etiologic pathogen is the fungus-like organism *Pythium insidiosum*. The disease has high mortality and morbidity rates. Use of antifungal drugs are ineffective against *P. insidiosum*, leaving radical surgery the main treatment option. Prompt treatment leads to better prognosis of affected individuals, and could be achieved by early and accurate diagnosis. Since pythiosis has been increasingly reported worldwide, there is a need for a rapid, user-friendly, and efficient test that facilitates the diagnosis of the disease. This study aims to develop an immunochromatographic test (ICT), using the bacterial protein A/G, to detect anti-*P. insidiosum* IgGs in humans and animals, and compare its diagnostic performance with the established ELISA. Eighty-five serum samples from 28 patients, 24 dogs, 12 horses, 12 rabbits, and 9 cattle with pythiosis, and 143 serum samples from 80 human

and 63 animal subjects in a healthy condition, with thalassemia, or with other fungal infections, were recruited for assay evaluation. Detection specificities of ELISA and ICT were 100.0%. While the detection sensitivity of ELISA was 98.8%, that of ICT was 90.6%. Most pythiosis sera, that were falsely read negative by ICT, were weakly positive by ELISA. In conclusion, a protein A/G-based ICT is a rapid, user-friendly, and efficient assay for serodiagnosis of pythiosis in humans and animals. Compared to ELISA, ICT has an equivalent detection specificity and a slightly lower detection sensitivity.

Key words: Pythiosis, *Pythium insidiosum*, Immunochromatography, Serodiagnosis.

Introduction

Pythiosis is a life-threatening infectious disease caused by the fungus-like, aquatic, oomycete organism *Pythium insidiosum*.[1–5] Most cases of pythiosis have been reported in humans, horses, dogs, cats, and cattle, but some other domestic and wild animals are also infected.[2,4,5] Tropical wetlands are the natural habitat of *P. insidiosum*, and the disease is likely to be acquired through the ingestion of contaminated water or direct contact with the pathogen to host surfaces.[6,7] Pythiosis has been found in Asian countries, that is, Thailand, Malaysia, and India.[4,8,9] However, the disease has been reported in the Americas (i.e., U.S.A., Costa Rica, Brazil),[10–12] Africa,[13] and parts of Australia and New Zealand.[14–16] Various forms of pythiosis have been observed, depending on the site at which the infection initiates, that is, artery, eye, skin, and gastrointestinal tract. Cutaneous/subcutaneous infection is the most common form of pythiosis in horses, whereas in dogs, gastrointestinal tract infection is more prevalent. In humans, *P. insidiosum* infections of arteries (vascular pythiosis) and eyes (ocular pythiosis) have been frequently reported.

Pythiosis has a high rate of mortality and morbidity.[2–5] Use of antifungal drugs are ineffective for the treatment of pythiosis. Extensive surgical removal of infected tissues is the main treatment option for cure. Prompt and effective treatment is required to promote better outcome for the affected individuals, and this could be achieved by early and accurate diagnosis of pythiosis. Several diagnostic methods are available for pythiosis.[2,4,5] Culture identification and PCR-based diagnostic assays are used for the direct detection of *P. insidiosum* in clinical samples.[8,17–22] However, these methods are time-consuming and require experienced personnel. In addition, culture identification often fails to isolate the organism from the infected tissue sample. Alternatively, serodiagnostic assays, including immunodiffusion (ID),[23,24] enzyme-linked immunosorbent assay (ELISA),[25–29] hemagglutination (HA),[30] and immunochromatographic test (ICT),[31] have been developed for detection of anti-*P. insidiosum* antibodies in serum samples. Among these assays, ELISA and ICT showed most favorable diagnostic performance for pythiosis.[28]

As alluded to earlier, pythiosis has been increasingly diagnosed in mammals, including humans and various domestic and wild animals worldwide.[2–5] There is a need for a serological test that could facilitate the diagnosis of *P. insidiosum* infections in these mammalian subjects. While ID is relatively slow and insensitive, HA has a poor diagnostic performance, and ELISA requires a multi-step procedure and expensive equipment, ICT appears to be a rapid, user-friendly, and efficient test format for serodiagnosis of pythiosis.[23–28,30,31] ICT has been developed to detect anti-*P. insidiosum* antibodies in serum samples particularly from human patients, and not that from other animals.[31] This limitation is, however, due to different reagents (i.e., host-specific anti-IgG antibodies) are needed to perform the test against sera obtained from different hosts. By using the bacterial protein A/G that binds various mammalian IgGs,[32,33] the present study aims to develop an ICT, using the bacterial protein A/G, to detect anti-*P. insidiosum* IgGs in humans and animals, and compare its performance with the established ELISA.[26,28,34]

Materials and methods

Serum samples

A total of 85 serum samples from 28 human patients (26 vascular, 1 ocular, and 1 cutaneous pythiosis), 24 dogs, 12 horses, 12 rabbits, and 9 cattle with pythiosis were recruited for ICT and ELISA analyses.[26,28,31,34] Diagnosis of pythiosis was based on: (i) culture identification of *P. insidiosum* from clinical specimens;[17] (ii) PCR-based assay or sequence homology analysis of *P. insidiosum* rDNA;[8,18,20] or (iii) detection of anti–*P. insidiosum* antibodies in serum samples by established serodiagnostic tests.[23–28,30,31,34] To serve as the control, a total of 143 serum samples were recruited from 80 human subjects (healthy blood donors [n = 62] and patients with thalassemia [n = 10], aspergillosis [n = 3], zygomycosis [n = 2], candidiasis [n = 1], cryptococcosis [n = 1], and histoplasmosis [n = 1]), 31 dogs (healthy dogs [n = 9], dogs with *Lagenidium giganteum* forma *caninum* infection

[n = 8], *Paralagenidium karlingii* infection [n = 6], zygomycosis [n = 3], aspergillosis [n = 1], blastomycosis [n = 1], cryptococcosis [n = 1], protothecosis [n = 1], and sporotrichosis [n = 1]), 12 healthy cattle, 10 healthy horses, and 10 healthy rabbits. All sera were kept frozen until use.

Antigen preparation

The *P. insidiosum* strain Pi-S, isolated from a Thai patient with pythiosis, was maintained on Sabouraud dextrose (SD) agar, and subcultured (at 37 °C) once a month until use. The antigen was prepared, using the protocol described by Krajaejun et al.[35] Briefly, several small pieces of SD agar containing growing *P. insidiosum* mycelium were cultured (at 37 °C for 10 days) with shaking (150 rpm) in a flask containing 200 ml of SD broth. The organism was killed by adding Thimerosal [final concentration: 0.02% (wt/vol)], and separated from cultured SD broth by filtration through a 0.22-μm pore size membrane (Durapore). To prepare the culture filtrate antigen (CFA), after adding protease inhibitors [PMSF (0.1 mg/ml) and EDTA (0.3 mg/ml)], the cell-free SD broth was concentrated ~80 fold using the Amicon 8400 apparatus and an Amicon Ultra-15 centrifugal filter (Millipore). Protein concentration of CFA was estimated by spectrophotometry. The CFA was stored at −20 °C until use.

Protein A/G-based immunochromatographic test

The protein A/G based ICT was produced at the Chulabhorn Research Institute and involved the following step:

(I) **Preparation of protein A/G colloidal gold conjugate:** Protein A/G (Prospec, Ness-Ziona, Israel) was coupled to a colloidal gold particle by pI-dependent passive adsorption. The 40-nm colloidal gold solution (Arista, Allentown, PA) was adjusted to pH 7.2 with 0.2 M Na_2CO_3 under gentle stirring. The gold suspension was divided into aliquots of 0.5 ml in 1.5 ml microcentrifuge tubes to which 30 μl of protein A/G (0.1 mg/ml) was added with gentle vortexing. The mixture was allowed to conjugate at room temperature for 30 min. The residual surface of the gold particle was blocked with 125 μl of 5% (wt/vol) casein dissolved in 5 mM sodium phosphate buffer (SPB) pH 7.4 for 15 min. The conjugation mixture was centrifuged at 6,000 × g at room temperature for 15 min, and the supernatant was discarded. The pellet was washed with 0.5% (wt/vol) casein and the suspended conjugated particle was centrifuged again under identical settings. After removing the supernatant, the pellet was re-suspended in a solution of 0.5% (wt/vol) casein containing 20% (wt/vol) sucrose in 5 mM SPB pH 7.4 to 2.5% of the original volume of colloidal gold suspension. The protein A/G colloidal gold conjugate (2.5 μl) was transferred to a piece of 2.5×4.0 mm glass fiber filter GF33; (Whatman Schleicher & Schuell, Dassel, Germany). The impregnated glass fiber was dried in a dehumidifier cabinet for 2 hours and was used to construct the ICT.

(II) **Immobilization of antigen and antibody onto a nitrocellulose membrane:** Immobilization of proteins on nitrocellulose membrane (NCmb) (AE99; Whatman Schleicher & Schuell, Dassel, Germany) was performed by passive physical adsorption in line pattern. A BioDot ZX1000^{TM} dispensing platform (BioDot, Irvine, CA) was used for this purpose. The transfer rate of the solution was set at 1 μl/cm. A 1.25×20 cm nitrocellulose membrane was lined along its length with 1 mg/mL CFA to form a test line and with 0.2 mg/ml normal rabbit IgG in 50 mM ammonium acetate buffer pH 4.5 to form a control line. The protein immobilized membrane was dried and blocked with 1% (wt/vol) BSA, 0.1% (wt/vol) trehalose in 10 mM SPB pH 7.4 and dried again in a dehumidifier cabinet.

(III) **Preparation of the sample pad:** The sample pad used in the ICT strip was made of paper (#903 Whatman Schleicher & Schuell, Dassel, Germany) previously immerged in 2% (wt/vol) Triton X-100, 0.05% (wt/vol) polyvinylpyrrolidone (PVP) in 50 mM Tris-HCl, pH 7.4 and dried in a dehumidifier cabinet.

(IV) **Composition and construction of the ICT strip:** The ICT strip system was assembled by utilizing five major components: the sample pad, the glass fiber impregnated with colloidal gold conjugate, the protein immobilized NC membrane, a wicking pad (3 MM chromatography paper, Whatman, Maidstone, England) and the plastic backing. The first 4 components were assembled with 1–2 mm overlap on the plastic backing support (G&L; San Jose, CA). The assembled card was then cut into 2.5 mm wide strips with a strip cutting machine (CM 4000 R; BioDot, Irvine, CA) (Figure 1).

(V) **Detection of anti-*P. insidiosum* antibodies in human and animal sera:** The ICT assay was carried out in a 96-well microtiter plate or a microtube. Human sera were diluted 1:10,000 (in 0.15 M PBS pH 7.4) and tested with ICT in Center #1 (Ramathibodi Hospital, Thailand), while animal sera were diluted 1:5,000 (in 0.15 M PBS pH 7.4) and tested with ICT in Center #2 (for samples from dogs; Louisiana State University, USA) and Center #3 (for samples from horses, dogs, cattle, and rabbits; Universidade Federal de Santa Maria, Brazil). The protein A/G-based ICT strip was dipped into a

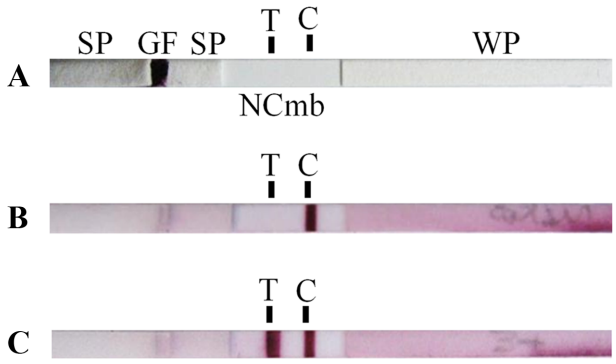

Figure 1. Schematic diagrams of the proteins A/G based immunochromatographic test (ICT) for detection of anti-*Pythium insidiosum* IgGs. Panel **A** (actual ICT strip) shows an untested ICT (either test or control line is not generated). Panel **B** depicts a negative result (only the control line is visible), whereas panel **C** exhibits a positive result (both test and control lines are visible). (Abbreviations: SP, sample pad; GF, glass fiber; WP, wicking pad; NCmb, nitrocellulose membrane; T, test line; and C, control line).

well containing 0.1 ml of each diluted serum sample in duplicate. The serum sample moved through the sample pad and the conjugate pad by capillary force. The mixture moved along the membrane immobilized with CFA acting as the test line. If anti-*P. insidiosum* antibody is present in the serum sample, the result is the formation of colored bands of colloidal gold conjugate at the test line and also the control line. On the other hand, a negative sample gives only one band at the control line. The developed signal of each ICT was read visually at 30 min. Detection sensitivity, detection specificity, and assay accuracy were calculated using the Microsoft EXCEL2013 program.

Enzyme-linked immunosorbent assay

ELISA for detection of anti-*P. insidiosum* antibodies in serum samples was carried out in three centers: (i) Department of Pathology, Faculty of Medicine, Ramathibodi Hospital, Mahidol University, Bangkok, Thailand (Center #1), using the ELISA protocol of Chareonsirisuthigul et al.[28] for testing human sera; (ii) Department of Veterinary Clinical Sciences, Louisiana State University, Baton Rouge, LA, USA (Center #2), using the ELISA protocol of Grooters et al.[26] for testing dog sera; and (iii) Departamento de Microbiologia e Parasitologia, Universidade Federal de Santa Maria, Santa Maria, RS, Brazil (Center #3), using the ELISA protocol of Santurio et al.[34] for testing sera from horses, dogs, cattle, and rabbits. The *P. insidiosum* antigen used for the ELISA performed in Center #1 was CFA (exoantigen),[28] while that used for the ELISA performed in Center #2 and Center #3 was soluble hyphal antigen.[26,34] The ELISA cut-off value was calculated based on the mean optical density (OD) of the control sera plus three SDs (Center #1 and #3), or the mean percent positivity of all control sample ODs (in relation to the strong positive control serum OD) plus three SDs (Center #2). Any samples with ELISA values above the cut-off were determined to be positive, while those below the cut-off were determined to be negative. Detection sensitivity, detection specificity, assay accuracy, and ELISA cut-off values were calculated using the Microsoft EXCEL2013 program.

Results

Development of a protein A/G-based ICT

An assembled protein A/G-based ICT is shown in Figure 1. CFA (crude protein extract from *P. insidiosum* strain Pi-S) and the commercially available normal rabbit IgGs were separately streaked as a straight line on a nitrocellulose membrane and served as "test" and "control" lines, respectively. Various IgG species in a human or animal serum samples were absorbed and migrated along the sample pad, and then the glass fiber, where complexes of IgGs and protein A/G conjugated with colloidal gold were formed. The IgG-protein A/G-colloidal gold complexes moved through the nitrocellulose membrane. The complexes containing anti-*P. insidiosum* IgGs captured the CFA blotted at the test line, and developed a purple signal of accumulated colloidal golds. The complexes lacking anti-*P. insidiosum* IgGs passed through the test line, without developing any signal. The normal rabbit IgGs, blotted at the control line, bound the protein A/G in the remaining complexes, and developed a purple signal.

After distribution of the ICT from Center #1 (Thailand) to Center #2 (USA) and Center #3 (Brazil), the assay can still function properly. After two years of storage at room temperature, the ICT can still effectively detect the anti-*P. insidiosum* IgGs in serum samples.

Performance comparison of ICT and ELISA

The established ELISA[26,28,34] and the protein A/G-based ICT had been independently performed to determine anti-*P. insidiosum* antibodies in sera from 108 humans, 55 dogs, 22 horses, 21 cattle, and 22 rabbits, with (n = 85) or without (n = 143; served as control) pythiosis, in the three centers: Center #1 (Department of Pathology, Ramathibodi Hospital, Mahidol University, Bangkok, Thailand), Center #2 (Department of Veterinary Clinical Sciences, Louisiana State University, Baton Rouge, LA, USA), and Center #3 (Departamento de Microbiologia e Parasitologia, Universidade Federal de Santa Maria, Santa Maria, RS, Brazil) (Table 1). The test results (i.e., ELISA value ranges, means, and cutoffs) and diagnostic performances (i.e., sensitivities,

Table 1. Diagnostic performances of ICT and ELISA tested against serum samples from humans (n = 108) or animals (n = 120), with and without pythiosis, in Center #1 (located in Thailand), Center #2 (USA), and Center #3 (Brazil).

Center	Host (Total sera)	Range of ELISA values (Mean)		ELISA cutoff value	ELISA			ICT		
		Pythiosis	Control		TP[a]/PC[b] (Sensitivity, %)	TN[c]/CC[d] (Specificity, %)	Accuracy (%)[e]	TP/PC (Sensitivity, %)	TN/CC (Specificity, %)	Accuracy (%)
1	Humans (108)	20.25–1.91 (13.47)	4.45–0.33 (1.55)	4.45	27/28 (96.4)	80/80 (100.0)	99.1	24/28 (85.7)	80/80 (100.0)	96.3
2	Dogs (44)	105.70–16.73 (62.38)	9.11–2.68 (5.01)	10.02	18/18 (100.0)	26/26 (100.0)	100.0	17/18 (94.4)	26/26 (100.0)	97.7
3	Dogs (11)	1.37–1.16 (1.25)	0.75–0.30 (0.47)	1.00	6/6 (100.0)	5/5 (100.0)	100.0	6/6 (100.0)	5/5 (100.0)	100.0
	Horses (22)	1.11–0.22 (0.80)	0.21–0.12 (0.17)	0.21	12/12 (100.0)	10/10 (100.0)	100.0	9/12 (75.0)	10/10 (100.0)	86.4
	Cattle (21)	0.37–0.17 (0.23)	0.16–0.09 (0.13)	0.16	9/9 (100.0)	12/12 (100.0)	100.0	9/9 (100.0)	12/12 (100.0)	100.0
	Rabbits (22)	0.33–0.19 (0.27)	0.15–0.09 (0.12)	0.18	12/12 (100.0)	10/10 (100.0)	100.0	12/12 (100.0)	10/10 (100.0)	100.0
1–3	Overall (228)	N/A[f]	N/A	N/A	84/85 (98.8)	143/143 (100.0)	99.6	77/85 (90.6)	143/143 (100.0)	96.5

[a]Number of cases with true positive (TP) results.
[b]Pythiosis cases.
[c]Number of cases with true negative (TN) results.
[d]Control cases.
[e]Accuracy (%), [(all cases with true positive and true negative results)/(all pythiosis and control cases)] × 100.
[f]N/A, not applicable.

specificities, and accuracies) of ELISA and the protein A/G-based ICT, according to host types (humans, dogs, horses, cattle, and rabbits), disease states (pythiosis or control), and assay-performing centers, are summarized in Table 1.

All control serum samples (n = 143) were tested negative by both the ELISA and protein A/G-based ICT. Among all 85 pythiosis sera, 77 samples were consistently tested positive by both serological assays. ELISA and ICT failed to detect anti-*P. insidiosum* antibodies in the same serum from a patient with ocular pythiosis (ELISA value: 1.91; ELISA cutoff: 4.45). Two patients with vascular pythiosis (ELISA values: 11.60 and 7.08), one patient with cutaneous pythiosis (ELISA value: 7.59), three horses with pythiosis (ELISA values: 0.26, 0.25, and 0.22; ELISA cutoff: 0.21), and a Center #2 dog with pythiosis (ELISA value: 16.73; ELISA cutoff: 10.02) were tested positive by ELISA but negative by ICT. One pythiosis serum from a human subject was weakly positive by ICT and ELISA (ELISA values: 6.28). Based on the results of all pythiosis and control sera, regardless of host types and assay-performing centers, ELISA showed 98.8% detection sensitivity, 100% detection specificity, and 99.6% accuracy, while ICT showed 90.6% detection sensitivity, 100% detection specificity, and 96.5% accuracy.

Discussion

The protein A/G-based ICT was successfully developed for the detection of specific anti-*P. insidiosum* IgGs in serum samples from humans and animals with pythiosis, which is a striking advantage over the previously-reported ICT that can detect only the IgGs from human subjects.[31] The diagnostic performance of ICT was compared to that of ELISA, a highly efficient assay established for the serodiagnosis of pythiosis in humans or animals.[26,28,34] Interpretation of ICT results depends on the presence or absence of the test line (which is subjectively read by the naked eye; Figure 1), while interpretation of ELISA results depends on an OD value above or below the cutoff (which was objectively quantitated by an ELISA plate reader). ICT and ELISA did not detect the anti-*P. insidiosum* IgG antibodies in the control sera from healthy individuals, as well as those from humans and animals with infections caused by other pathogens, including those share microscopic morphologies with *P. insidiosum* (i.e., *Lagenidium*, *Paralagenidium*, *Aspergillus*, and Zygomycetes) (Table 1). This finding indicates that both ELISA and ICT had no cross-reactivity with other pathogens and thus provides equivalently high detection specificity (100%).

Regardless of the sources of the sera tested, the overall detection sensitivity of ICT was considered high (~91%),

although slightly lower than that of ELISA (~99%; Table 1). This was due to some serum samples from several proven cases of human (n = 4), equine (n = 3), and canine (n = 1) pythiosis, being read negatively by ICT (i.e., no visible test line), but marginally positive by ELISA (i.e., ELISA values that were slightly above the cutoff; Table 1). The subjective nature of result interpretation could explain the limited detection sensitivity of ICT, especially when fewer anti-*P. insidiosum* antibodies were present in the serum sample, as indicated by a low ELISA value. The possibility of the presence of anti-protein A/G antibodies in the serum, that inhibits the formation of IgG-protein A/G-colloidal gold complexes, and leads to an absence of the test line, is unlikely. This can be explained by the fact that the ICT control line, generated by complex formation of the normal rabbit IgGs and the protein A/G conjugated with colloidal gold (Figure 1), was strongly developed in all serum samples tested, indicating that there was no anti-bacterial protein A/G antibodies in the samples. The reason for the false negative results was most likely due to the lower detection sensitivity of the ICT, as compared to that of ELISA. Poor host antibody responses can be observed in localized infections of the eye,[30,31] and could explain the failure of ICT and ELISA to detect anti-*P. insidiosum* antibodies in the serum from patient with ocular pythiosis. Therefore, the use of neither ICT nor ELISA is recommended for making a diagnosis of ocular pythiosis.

ELISA and ICT showed high accuracy (99.6% and 96.5%, respectively; Table 1), indicating that both assays reliably reported the true positive and true negative results. Here, we showed that ELISA is highly sensitive for the diagnosis of pythiosis, which was consistent with the reports of other investigators.[25–28,34] However, ELISA has a long turnaround time (>3 hr), and to perform this multi-step assay, it requires a specific secondary antibody (for each host type), experienced personnel, and special equipment. Such limitations of ELISA could be addressed by the successful development of the protein A/G-based ICT, which appears to be rapid (shorter turnaround time: ~30 min), highly-sensitive (91%), and easy-to-use assay that can facilitate serodiagnosis of pythoisis, especially in nonreference laboratories.

In conclusion, an ICT has been successfully developed for the serodiagnosis of humans and animals with pythiosis. ICT has a high detection sensitivity (91%), detection specificity (100%), and accuracy (97%). Yet, it was designed and manufactured to be a rapid, user-friendly, and efficient test. The current ICT has a long shelf storage life (at least two years), and it can be distributed worldwide, without effecting its performance. ICT could facilitate the diagnosis of pythiosis in most cases. However, if ICT is read negative in a suspected case of pythiosis, further analysis using a more sensitive assay (i.e., ELISA) is recommended.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

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