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Brief communication (Original)

PCR amplification of a putative gene for exo-1,3-β-glucanase to identify the pathogenic oomycete *Pythium insidiosum*

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Background: *Pythium insidiosum* is the etiologic agent of pythiosis, a life-threatening infectious disease. Diagnosis of pythiosis is difficult and often delayed. Early diagnosis can lead to prompt treatment, and therefore a better prognosis for patients with pythiosis. Molecular diagnostic techniques are useful if microbiological and immunological assays are not available, or in cases of suspected pythiosis that test negative by other methods. So far, PCR identification of *P. insidiosum* has been largely relied on amplification of the rDNA region.
Objective: To evaluate the diagnostic performance of Dx3 and Dx4 primers specific for a putative gene for exo-1,3-β-glucanase (*PinsEXO1*), which encodes a specific immunogen of *P. insidiosum*, for rapid single-round PCR identification of *P. insidiosum*, in comparison with the previously-reported rDNA-specific primers, ITSpy1 and ITSpy2.
Materials and Methods: Genomic DNA (gDNA) from 35 *P. insidiosum* isolates and 48 control organisms were prepared to evaluate the diagnostic performance of the *PinsEXO1*- and rDNA-specific primers.
Results: When amplifying the control gDNA by using the Dx3/4 and ITSpy1/2 primer sets, no PCR product was observed, indicating that both primer sets had 100% detection specificity. When amplifying the *P. insidiosum* gDNA, the Dx3/4 primers provided an expected 550-bp amplicon for all 35 isolates, while the ITSpy1/2 primers provided an expected 230-bp amplicon for only 32 isolates. Thus, detection sensitivity of the Dx3/4 and ITSpy1/2 primer sets were 100% and 91%, respectively.
Conclusion: By using the Dx3/4 primers, *PinsEXO1* was an alternative, efficient, and novel PCR target for rapid single-round PCR identification of *P. insidiosum*.

Keywords: Diagnosis, oomycete, PCR, pythiosis, *Pythium insidiosum*

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

Early diagnosis and prompt treatment can minimize the morbidity and mortality rates of pythiosis. Definitive diagnosis of pythiosis can be made by culture identification [5], immunohistological assays [6-8], and serodiagnostic tests [9-20]. Some limitations have been observed in these assays. For example, the culture identification technique is a time consuming, requires

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experience, and is relatively insensitive. Serodiagnostic tests usually fail to detect anti-*P. insidiosum* antibody in patients with ocular pythiosis. A tissue sample (i.e. paraffin-embedded tissue) is often the only available source of specimen for diagnosis of pythiosis. In such case, diagnosis using an immunohistological assay is more suitable. However, the immunohistological assay requires a specific rabbit anti-*P. insidiosum* antibody, which is not routinely available. Moreover, cross reactivity with some other pathogenic fungi (i.e., *Conidiobolus* and *Fusarium* species) compromises the specificity of the assay [7, 21].

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

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

Recently, we reported a putative gene for exo-1,3-β-glucanase (*PinsEXO1*), which encodes a specific immunoreactive protein of *P. insidiosum* [29]. The *PinsEXO1*-coding sequences of all 22 *P. insidiosum* isolates tested are conserved, suggesting that the gene can be a potential PCR target for *P. insidiosum* identification. In the present study, we aimed to design and evaluate *PinsEXO1*-specific primers Dx3 and Dx4 for rapid single-round PCR identification of *P. insidiosum*, in comparison with the rDNA-specific primers, ITSpy1 and ITSpy2.

Materials and methods

Microorganisms

Thirty-five clinical (n = 31) and environmental (n = 4) isolates of *P. insidiosum* were used for genomic DNA (gDNA) preparation. Identity of all *P. insidiosum* isolates was confirmed by culture identification and zoospore induction [5]. Forty-eight culture-proven isolates of various fungi (**Table 1**) from the Clinical Microbiology Laboratory, Department of Pathology, Faculty of Medicine, Ramathibodi Hospital, Mahidol University, were also used for gDNA preparation (**Table 1**). All microorganisms were maintained on Sabouraud dextrose agar until use. This study was approved by the Committee on Human Rights Related to Research Involving Human Subjects at the Faculty of Medicine, Ramathibodi Hospital, Mahidol University. Informed consent from the patients providing the samples from which the isolates were originally obtained was waived because these patients are unknown to the current investigators and the data was analyzed anonymously, nor is it the intention of the authors to identify any patients retrospectively.

Genomic DNA extraction

Extraction of *P. insidiosum* gDNA was performed using a modification of the method reported by Aljanabi et al. [30]. Briefly, a hyphal mat (~100 mg), harvested from 7-day culture in Sabouraud dextrose broth, was ruptured with glass beads (diameter, 710–1,180 μm; Sigma, St. Louis, MO, USA) in the presence of 400 μL of the salt homogenizing buffer (0.4 M NaCl, 10 mM Tris-HCl (pH 8.0), 2 mM EDTA), using TissueLyzer MM301 (Qiagen, Hilden, Germany). SDS (17% (wt/vol); 45 μL) and proteinase K (20 mg/mL; 8 μL) were added to the cell lysate and incubated at 56°C for overnight. NaCl (6 M; 0.3 mL) was then added. The mixture was briefly vortexed and centrifuged (10,000 ×*g* for 30 min). Supernatant obtained was added to an equal volume of isopropanol, mixed, stored at –20 °C for 1 h, and centrifuged (10,000 ×*g* for 20 min). The pellet was washed with 70% ethanol, air dried, and dissolved in 50–100 μL sterile water.

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

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

[a]*P. insidiosum* isolated from patients with vascular pythiosis (n = 14), patients with ocular pythiosis (n = 10), patients with cutaneous pythiosis (n = 3), patients with other forms of pythiosis (n = 2), isolated from animals (n = 2), and isolated from environment (n = 4)
[b]*Candida* species were *C. albicans* (n = 2), *C. tropicalis* (n = 2), *C. parapsilosis* (n = 2), *C. guilliermondii* (n = 2), *C. rugosa* (n = 1), *C. lusitaniae* (n = 1), *C. laurentii* (n = 1), and *C. dubliniensis* (n = 1)
[c]*Aspergillus* species were *A. flavus* (n = 1), *A. terreus* (n = 1), and *A. fumigatus* (n = 1)
[d]*Trichophyton* species were *T. rubrum* (n = 3), *T. mentagrophytes* (n = 1)
[e]*Trichosporon* species were *T. asahii* (n = 1), *T. mucoides* (n = 1)

Extraction of gDNA of the fungal controls (**Table 1**) was performed using a modification of the method reported by Muller et al. [31] and Niu et al. [32]. Briefly, portions of hyphal colony (about 50–100 mg) were harvested from actively growing (5–7-day-old) cultures on Sabouraud dextrose agar, and transferred to a 1.7 mL tube. Lysis buffer (600 µL of 100 mM Tris-HCl (pH 8.0), 50 mM EDTA, 500 mM NaCl, 0.2% 2-mercaptoethanol (v/v), and 0.3% SDS) was added to each sample, vortexed vigorously with glass beads (diameter, 710–1,180 µm; Sigma), and incubated at 65°C for 3 h. The cell lysate was centrifuged at 10,000 ×*g* for 10 min. DNA was extracted with an equal volume of phenol:chloroform (1:1). To precipitate DNA, 0.1 volume of 3 M potassium acetate buffer (pH 5.2) and 2 volumes of absolute ethanol were added to the mixture, incubated at –20°C for 30 min, and centrifuged at 10,000 ×*g* for 10 min. The DNA pellet was then washed with 70% ethanol, dried, and resuspended in 40 µL of TE.

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

Polymerase chain reaction

The PCR amplifications were performed in a 25 µL mixture comprising 100 ng gDNA template, 10mM each primer, 1.5 mM $MgCl_2$, 0.2 mM dNTP mixture

(Promega, Madison, WI, USA), and 0.5 UI Taq-polymerase (Fermentas, Hanover, MD, USA) in 1× Taq-polymerase buffer. Amplification reactions were conducted in a Mastercycler-Pro thermal cycler (Eppendorf, Westbury, NY, USA) with the following settings: predenaturation at 94°C for 3 min, 25 cycles of denaturation at 94°C for 45 s, annealing at 55°C for 1 min, and extension at 72°C for 1 min, followed by the final extension at 72°C for 7 min. Three pairs of primers were used to amplify all extracted gDNAs: (i) the fungal universal primer ITS1 (52 -TCCGTAGG-TGAACCTGCGG-32) and ITS4 (52 -TCCTCCGC-TTATTGATATGC-32) [21]; (ii) the rDNA-specific primer ITSpy1 (5′-CTGCGGAAGGATCATTACC-3′) and ITSpy2 (5′-GTCCTCGGAGTATAGATCAG-3) [18]; and (iii) the *PinsEXO1*-specific primer Dx3 (5-GCGAGTTCTGGCTCGACTTTA-3′) and Dx4 (5′-ACAAGCGCCAAAAAGTCCCA-3′) designed by using the Primer-BLAST program [33], and the *PinsEXO1* accession number GU994093.1 [29]. A negative control (no template) was included in each round of PCR assays. The GeneRuler 100-bp plus DNA ladder (Fermentas) was used to indicate molecular size. Size and amount of each PCR product were analyzed by 1% agarose gel electrophoresis, and image captured by Molecular Imager Gel Doc XR+ (Bio-Rad, Hercules, CA, USA).

DNA sequencing

The primer Dx3 used to sequence the *PinsEXO1*-derived amplicon, and primer ITSpy1 was used to sequence the rDNA-derived amplicon. PCR products were purified using a NucleoSpinR Gel and PCR clean-up kit (NucleoSpin, Macherey-Nagel, Duren, Germany). Direct sequencing was performed using a Big Dye Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Automated sequencing was performed and analyzed using an ABI 3100 Genetic Analyzer and the Applied Biosystems Sequencing software. For sequence homology analysis, each sequences obtained was Blasted against the NCBI nucleotide database (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Results

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

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

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

Discussion

Molecular diagnostic techniques for pythiosis are useful if microbiological and immunological assays are not available, or in suspected cases that test negative by other methods. So far, PCR identification of *P. insidiosum* has largely relied on amplification of the rDNA region [22]. As an alternative assay, we evaluated diagnostic performance of the PCR primers, Dx3 and Dx4, which target *PinsEXO1* of *P. insidiosum* [29], in comparison with the previously-reported rDNA-specific primers, ITSpy1 and ITSpy2 [18]. The primer Dx3 and Dx4 were designed by using the well-established program called Primer-BLAST [33], which is publicly available at the NCBI website (http://www.ncbi.nlm.nih.gov/tools/primer-blast).

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

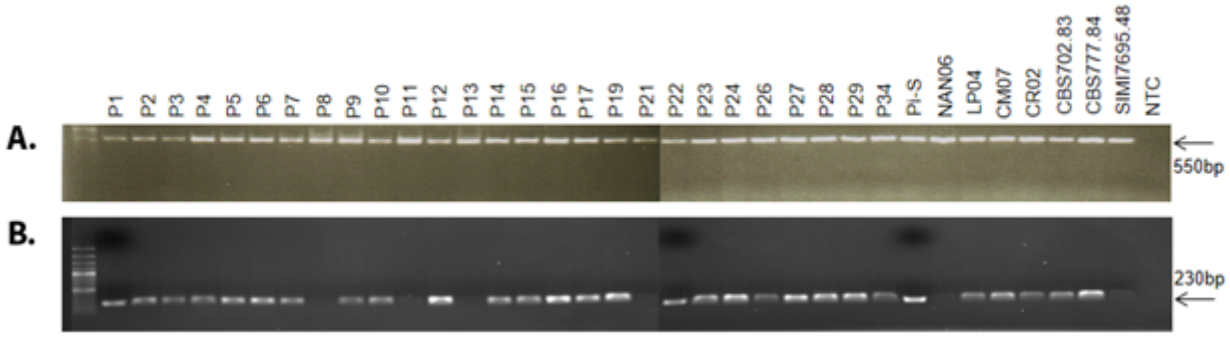

Figure 2. Gel electrophoresis showing the PCR products amplified from gDNA of the 35 *P. insidiosum* isolates by using: (**A**) the Dx3/4 primers, which target the putative exo-1,3-β-glucanase gene of *P. insidiosum* or *PinsEXO1* (amplicon size, 550bp); and (**B**) the ITSpy1/2 primers, which target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

Based on a given DNA target sequence (e.g. *PinsEXO1*), the Primer-BLAST program selects the most suitable primer set and uses the BLAST and global alignment algorithm to evaluate the selected primers for any cross annealing against all genes deposited in the NCBI nucleotide database. Thus, the resulting primers (Dx3 and Dx4) should be specific to the target sequence.

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

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

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

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