Evaluation for the Clinical Diagnosis of *Pythium insidiosum* Using a Single-Tube Nested PCR

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Abstract Pythiosis is a rare infectious disease caused by *Pythium insidiosum*, which typically occurs in tropical and subtropical regions. The high mortality rate may be in consequence of the lack of diagnosis. The objective of this study was to evaluate reliability of a new single-tube nested PCR for detection of *P. insidiosum* DNA. A total of 78 clinical isolates of various fungi and bacteria, 106 clinical specimens and 80 simulated positive blood samples were tested. The developed primer pairs CPL6–CPR8 and YTL1–YTR1 are located on 18S subunit of the rRNA gene of *P. insidiosum*. The specificity, negative and positive predictive values were 100, 100 and 87.5 %, respectively, as compared with direct microscopy and cultivation. The detection limit of the single-tube nested PCR was 21 zoospores corresponding to 2.7 pg of the DNA. The results demonstrate that the new single-tube nested PCR offers a highly sensitive, specific and rapid genetic method for detecting *P. insidiosum*.

Keywords *Pythium insidiosum* · Pythiosis · Single-tube nested PCR · 18S rRNA

Introduction

Pythiosis is an emerging and life-threatening infectious disease caused by the oomycete *Pythium insidiosum*, which can develop in humans and animals [1–4]. The infective stage of *P. insidiosum* is a biflagellate zoospore [1]. Human pythiosis occurs predominantly in tropical areas of the world and particularly in Thailand [1, 5, 6]. There, the first documented case of human pythiosis was reported in 1985 [7]. According to clinical signs, human pythiosis can be divided into four types: cutaneous/subcutaneous, vascular, ocular and disseminated. The first type is characterized by chronic swelling, painful subcutaneous granulomatous...
infiltration and ulceration, usually located in the face or legs [8]. Chronic arthritis in the lower extremities resulting in arterial occlusion and gangrenous ulceration of feet or legs is typical for vascular pythiosis [9]. The ocular form is usually manifested as corneal ulcers or keratitis. As a result of all these forms of infection, P. insidiosum can spread via the bloodstream to various internal organs or organ systems such as the gastrointestinal tract, brain, liver, kidney or rhinosinus [10]. Currently, the diagnosis of pythiosis is based on microscopy, culture, detection of antibodies and molecular genetic techniques [9, 11–13]. However, microscopy cannot distinguish zygomycetes because of the coenocytic form of the mycelium [7]. Culture is time-consuming, and obtaining infected tissue samples may be difficult [1]. Because of low antibody response, false-negative results frequently occur in serological tests, particularly in ocular pythiosis [11]. Nested PCR has been developed for the diagnosis of pythiosis using the internal transcribed spacer 1 (ITS1) of the gene for rRNA [14]. Although it is highly sensitive, the main problem of this PCR is a high risk of contamination as the product of the first reaction needs to be transferred into another tube for the second reaction. The purpose of this study was to solve this problem by developing a nested PCR for detecting P. insidiosum in a single tube and to evaluate its reliability using various clinical specimens, including simulated positive blood samples and clinical isolates of bacteria and fungi.

Materials and Methods

Clinical Isolates

The study comprised 34 isolates of P. insidiosum as specified in Table 1, 29 fungal isolates (Aspergillus fumigatus, Aspergillus flavus, Aspergillus niger, Basidiobolus ranarum, Candida albicans, Candida tropicalis, Cladophialaphora carriionii, Curvularia spp., Exophiala jeanselmei, Filobasidiella neoformans, Fusarium spp., Hortaea werneckii, Lichtheimia spp., Microsporum gypseum, Mucor spp., Penicillium spp., Rhizoctonia spp., Rhizopus spp., Saccharomyces cerevisiae, Scedosporium apiospermum, Syncephalastrum spp., Talaromyces marneffei, Trichophyton spp., Trichophyton concentricum, Trichophyton rubrum, Trichophyton schoenleinii, Trichophyton tonsurans, Trichophyton violaceum and Trichosporon spp.), 10 bacterial isolates (Burkholderia pseudomallei, Corynebacterium spp., Enterococcus spp., Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Salmonella typhi, Staphylococcus aureus, Streptococcus pyogenes and Streptococcus viridans) and 5 isolates of non-insidiosum Pythium spp. (P. aphanidermatum, P. deliene, P. grandisporangium, P. middletonii and P. ultimum). All microorganisms were obtained from patients of the Siriraj Hospital, Mahidol University, Bangkok, Thailand. All isolates were identified based on conventional microbiological methods and then stored in skim milk (Oxoid, UK) 100 mg/ml with 33 % glycerol at −20 °C.

Clinical Specimens

One hundred and six clinical specimens from patients with suspected fungal infection obtained from a routine mycology laboratory in the Sirinarind Hospital, Khon Kaen University, Khon Kaen, Thailand were evaluated prospectively from May 2011 to February 2012. They included pus (n = 27), tissue biopsies (n = 19), blood (n = 10), bone marrow (n = 9), lymph nodes (n = 6), peritoneal dialysis fluid (n = 6), sputum (n = 5), bronchial washing (n = 5), tracheal secretion (n = 5), urine (n = 5), cerebrospinal fluid (n = 4), synovial fluid (n = 2), ascitic fluid (n = 2) and pleural fluid (n = 1). In addition, 80 simulated positive blood specimens were prepared from normal blood samples. For this purpose, each blood sample was mixed with zoospores of P. insidiosum so that each sample contained 1.15 × 10^6 zoospores ml/l.

Evaluation by Phenotypic Methods

All clinical specimens were evaluated microscopically in 20 % potassium hydroxide preparation for the presence of P. insidiosum hyphae. Culture was performed, with each specimen being inoculated on two Sabouraud dextrose agars (SDA; Oxoid, UK), two Mycosel agars (MCA; BD Diagnostics) and one blood agar (Oxoid, UK) for detection of P. insidiosum growth. One SDA and MCA each were incubated at 25 °C and the other media at 37 °C. All agars were evaluated for the P. insidiosum growth until 30 days. The suspected colonies were identified as P. insidiosum by induction of zoospores [15]. Results of these
phenotypic methods were then compared with a single-tube nested PCR for sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV).

DNA Extraction for the PCR

DNA from all clinical and simulated positive specimens was extracted with the NucleoSpin Tissue kit.
(Macherey–Nagel, Germany) and QIAamp DNA Mini Kit (Qiagen) according to the manufacturers’ instructions. All fungal isolates were cultured in 250 ml of Sabouraud dextrose broth (Oxoid, UK) and incubated at a room temperature for 7 days with shaking (150 rpm) in a rotary shaker (PSU 2T plus, BioSan, Latvia). Fungal mycelia were filtered, washed twice with deionized water and frozen at −20 °C until used.

Bacterial isolates were cultured in 3 ml of Luria broth (Oxoid, UK) and incubated at 37 °C for 16–18 h with shaking (200 rpm). Then, cultures were transferred to a 1.5 ml microtube, centrifuged and stored at 4 °C. For cell disruption, approximately 30 mg of frozen fungal mycelia and 0.14–1.21 g of the bacterial pellet were rubbed in liquid nitrogen until a fine powder. The bacterial powder was then suspended in a lysis buffer (25 mM Tris–HCl pH 8, 10 mM EDTA pH 8, 100 mM NaCl). DNA was extracted from the bacterial suspensions and powders from fungal mycelia as described by Sambrook and Russell [16]. Purity of isolated DNA was calculated using a spectrophotometer at OD 260 and 280.

Single-Tube Nested PCR

The single-tube nested PCR was performed using outer primers CPL6 (5′-GAC ACA GGG AGG TAG TGA CAA TAA ATA-3′) and CPR8 (5′-CTT GGT AAA TGC TTT CGC CT-3′), and inner primers YTL1 (5′-CTT TGA GTG TGT TGC TAG GAT G-3′) and YTR1 (5′-CTG GAA TAT GAA TAC CCC CAA C-3′) designed by ourselves using the GenBank database. The primers are located on the 18S subunit of the rRNA gene of P. insidiosum (GenBank accession no. AF442497). The position of the newly designed primer is shown in Fig. 1. The reaction mixture in a total volume of 50 μl contained 1 U of Platinum Taq DNA polymerase (Invitrogen), 0.2 mM MgCl2, 25 pmol of each primers and 0.2 mM dNTPs. Amplification was performed in a thermal cycler (Mastercycler Personal, Eppendorf International) using the following temperature cycles: initial denaturation at 95 °C for 5 min, followed by 30 cycles (denaturation at 95 °C for 1 min, annealing at 68 °C for 1 min and extension at 72 °C for 1 min), another 30 cycles (denaturation at 95 °C for 1 min, annealing at 57 °C for 1 min and extension at 72 °C for 1 min) and final extension for 10 min at 72 °C. Amplicons were visualized under UV light using a manual documentation system (InGenius3, Syngene, USA) after electrophoresis of 10 μl of the reaction solution in 2 % UltraClean agarose gel (Mo Bio Laboratories) containing the SYBR Gold nucleic acid gel stain (Invitrogen).

Evaluation of PCR Sensitivity

To determine the lowest detection limit of our nested PCR, seven 10-fold dilutions were prepared from simulated positive blood specimen. Then, 180 μl of each dilution containing 2.07 × 105–2.07 × 10−1 zoospores was used for nested PCR. As a control, DNA of P. insidiosum hyphae was also prepared as 10-fold serial dilution from 1 ng to 1 fg, and then, it was also amplified by a single-tube nested PCR. This study was approved by the Ethical Committee on Human Experimentation in Khon Kaen University in accordance with the Declaration of Helsinki (HE 541111).

Results

If P. insidiosum DNA was presented in clinical material, four amplicons sized 512, 452, 340 and 240 bp were detected by our nested PCR.

Evaluation of PCR Specificity Within Clinical Isolates

Four amplicons were detected in all 34 P. insidiosum isolates. Only one genus-specific product (512 bp) was found in five non-insidiosum Pythium spp. as demonstrated in Fig. 2. No product was found after amplification of DNA from all the remaining 29 fungal and 10 bacterial isolates. Therefore, our single-tube nested PCR had 100 % specificity within the group of clinical microorganisms tested in this study.

Evaluation of PCR Specificity in Clinical Specimens

Based on positivity of phenotypic methods, seven pus specimens from corneal ulcers were evaluated as positive within all tested clinical specimens. From that, three specimens were microscopically negative; culture and zoospore induction were positive in all seven specimens. Moreover, as demonstrated in
Table 2, one more pus specimen, also from a corneal ulcer, which was negative in both phenotypic methods, was positive in our single-tube nested PCR.

Evaluation of PCR Sensitivity

As shown in Fig. 3, the lowest detection limit in diluted simulated positive blood specimen was 21 zoospores, which corresponded to 2.7 pg of DNA. The lowest detection limit of serially diluted DNA isolated from *P. insidiosum* hyphae was 1 pg.

Evaluation of PPV and NPV of PCR in Clinical Specimens

These results are summarized in Table 3. Based on results of phenotypic methods, 7 specimens were evaluated as positive for *P. insidiosum*. Using our single-tube nested PCR, DNA of *P. insidiosum* was detected in 8 specimens, whereas 7 of them were positive by phenotypic methods. Therefore, NPV and PPV were 100 and 87.5 %, respectively.

Discussion

In our set of clinical specimens, one patient was found to be positive in single-tube nested PCR, but negative in phenotypic tests. In our study, the criterion for detecting pythiosis was positivity in one of two phenotypic methods. However, Krajaejun et al. [9] defined the criteria for pythiosis as culture positivity, seropositivity or clinical manifestation. The patient who had negative results of phenotypic tests and was
positive in nested PCR had symptoms of ocular pythiosis evaluated by a clinician. The reason why phenotypic methods could not confirm pythiosis in this case was probably their low sensitivity [17]. So, this finding demonstrates the superiority of PCR over phenotypic methods from the sensitivity point of view. The detection limit of our nested PCR in this study (2.7 pg of DNA in relation to 21 counted zoospores) allows us to suppose that one zoospore contains 128.57 fg of DNA. However, our experiments showed that the lowest detected amount of DNA from *P. insidiosum* isolated from its mycelium was 1 pg of DNA, corresponding to about eight zoospores. According to this detection limit, one zoospore could contain 48–129 fg of DNA. This is the first report which tried to estimate the amount of DNA in *P. insidiosum* zoospores. Hussain et al. [18] reported a detection limit of PCR for *Phytophthora infestans* of 0.5 pg, which corresponded to four zoospores; in that case, one zoospore should contain 125 fg of DNA. The DNA content is similar to data about plant pathogenic organisms from the order *Peronosporales* (*Oomycota*) ranging from 46 to 163 fg [19].

The newly designed, genus-specific outer set of primers CPL6 and CPR8 amplified a 512 bp fragment from a conserved part of the 18S subunit of the rRNA gene and species-specific inner primers YTL1 and YTR1 amplified a 240 bp fragment from variable region of this subunit were firstly evaluated in this study. Although finally four PCR products were seen in a single-tube nested PCR, the test showed excellent sensitivity and specificity. The fragment of 452 bp was the product of CPL6 and YTR1 primers, and the 300 bp fragment was amplified by the YTL1 and CPR8 primers. Four PCR products were detected in all 34 strains of *P. insidiosum* and in clinical samples from patients with pythiosis. A sensitivity of 100 % and a low detection limit allow us to recommend our single-tube nested PCR as a

### Table 2

Comparison of *P. insidiosum* detection in positive clinical specimens by phenotypic methods and a single-tube nested PCR

| Specimen no. | Phenotypic method | Genotypic method |
|--------------|-------------------|------------------|
|              | Microscopy        | Culture and zoospore induction | Single-tube nested PCR |
| 1            | +                 | +                | +                           |
| 2            | +                 | +                | +                           |
| 3            | +                 | +                | +                           |
| 4            | −                 | +                | +                           |
| 5            | −                 | −                | +                           |
| 6            | +                 | +                | +                           |
| 7            | −                 | +                | +                           |
| 8            | −                 | +                | +                           |

All specimens were pus from corneal ulcers

### Table 3

Comparison of phenotypic test results with a single-tube nested PCR

| Phenotypic tests (microscopy in 20 % KOH and culture) | Total |
|------------------------------------------------------|-------|
| Positive                                             | 7     |
| Negative                                             | 0     |
| Total                                                | 7     |

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useful tool for laboratories, which need to detect *P. insidiosum* in clinical material. Pythiosis is a rare but fatal infection in humans. Krajaejun et al. [8] reported *P. insidiosum* in clinical material. Pythiosis is a rare but useful tool for laboratories, which need to detect *P. insidiosum* in clinical material. Pythiosis is a rare but fatal infection in humans. Krajaejun et al. [8] reported *P. insidiosum* in clinical material.

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