International Journal of Medical Microbiology 304 (2014) 321–326

Contents lists available at ScienceDirect

International Journal of Medical Microbiology

journal homepage: www.elsevier.com/locate/ijmm

Molecular detection of *Pythium insidiosum* from soil in Thai agricultural areas

Nongnuch Vanittanakom[a,*], Jidapa Szekely[b], Sophit Khanthawong[c], Pritsana Sawutdeechaikul[a], Pramote Vanittanakom[d], Matthew C. Fisher[e]

[a] *Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand*
[b] *Faculty of Medical Technology, Prince of Songkhla University, Songkhla, Thailand*
[c] *Department of Microbiology and Parasitology, Faculty of Medical Science, Naresuan University, Phitsanulok, Thailand*
[d] *Faculty of Medicine, University of Phayao, Phayao, Thailand*
[e] *Department of Infectious Disease Epidemiology, Norfolk Place, Imperial College, London, UK*

ARTICLE INFO

Article history:
Received 6 October 2013
Received in revised form 8 November 2013
Accepted 25 November 2013

Keywords:
Pythium insidiosum
Pythiosis
Molecular detection
Nested PCR
Soil

ABSTRACT

Pythium insidiosum is an aquatic fungus-like organism in the kingdom Stramenopila that causes pythiosis in both humans and animals. Human pythiosis occurs in ocular, localized granulomatous subcutaneous and systemic or vascular forms. Individuals whose occupations involve exposure to aquatic habitats have an elevated risk of contracting pythiosis. Previously, we reported the first successful isolation of *Pythium insidiosum* from aquatic environmental samples by culture including confirmation using molecular methods. In this study, we show that *P. insidiosum* inhabits moist soil environments in agricultural areas. A total of 303 soil samples were collected from 25 irrigation sources in the areas nearby the recorded home addresses of pythiosis patients residing in northern provinces of Thailand. *P. insidiosum* DNA was identified directly from each soil extract by using a nested PCR assay and subsequent phylogenetic analysis of the ribosomal intragenic spacer region. *P. insidiosum* DNA could be detected from 16 of the 25 soil sources (64%). Conventional culture methods were also performed, however all samples exhibited negative culture results. We conclude that both irrigation water and soil are the natural reservoirs of *P. insidiosum*. In endemic areas, the exposure to these environmental reservoirs should be considered a risk factor for hosts susceptible to pythiosis.

© 2013 Elsevier GmbH. All rights reserved.

Introduction

Pythium insidiosum is classified as an oomycete in the kingdom Stramenopila, phylum Oomycota, order Peronosporales and family Pythiaceae (Alexopoulos et al., 1996; Dick, 2001). *P. insidiosum*, similar to other species in the genus *Pythium*, grows on agar medium as a fungal-like colony with nonseptate hyphae or sparsely septate form. The organism has been previously referred to as one of the members in the Kingdom Fungi. Later, strong phylogenetic evidence revealed that the microorganisms in the genus *Pythium* (the Straminipilans) are not fungi, but protistal microbes very closely related to algae and plants (Baldauf et al., 2000; Martin, 2000; Dick, 2001). Most *Pythium* species are important plant pathogens. *P. insidiosum* is the only species of the genus that can cause disease in animals and humans. The organism was described in detail by De Cock et al. (1987) and is characterised by having nonseptate filaments and motile zoospores. The zoopores can be induced in an induction water medium and can be trapped from aquatic environments by baiting with plant leaves, rabbit, horse or human hair (Mendoza and Prendas, 1988; Supabandhu et al., 2008; Vanittanakom et al., 2004).

P. insidiosum causes pythiosis, a disease in both animals and humans. Animal pythiosis, also known as swamp cancer, was initially reported in the 1970s as a subcutaneous disease in horses in New Guinea (Auswick and Copeland, 1974). Infection in humans caused by *P. insidiosum* is presumably acquired through the attachment of zoospores to injured tissue and hair. The disease exhibits a variety of clinical pathologies, including subcutaneous, vascular, systemic, or ophthalmic types. The systemic or vascular forms usually occur in thalassemic or leukemic patients (Laohapensang et al., 2005; Prasertwitayakij et al., 2003; Thianprasit et al., 1996; Wanachiwanawin et al., 1993) and molecular diagnosis of the disease can be undertaken using ELISA, immunoblot or PCR – based methods (Vanittanakom et al., 2004; Krajaejun et al., 2002). Human pythiosis in southeast Asia commonly occurs during the rainy season and summer, with particularly high frequencies observed during the peak of rice-farming. In the United States, equine

* Corresponding author. Tel.: +66 53945332.
E-mail addresses: drnongnuch@gmail.com, nvanitta@med.cmu.ac.th (N. Vanittanakom).

1438-4221/$ – see front matter © 2013 Elsevier GmbH. All rights reserved.
http://dx.doi.org/10.1016/j.ijmm.2013.11.016

pythiosis develops in horses that graze for a long period of time in stagnant water and canine pythiosis cases commonly occur when dogs ingest, or swim in, contaminated water. Some pythiosis cases however involve humans and canines that have never been exposed to stagnant water; instead, they had contact with soil or had skin or eye damage by plant leaves (Krajaejun et al., 2004, 2006; Dykstra et al., 1999; Fischer et al., 1994). These cases indicate that infection by *P. insidiosum* may occur not only *via* water-borne zoospores, but also through resting oospores or hyphae found in soil or plants (Salas et al., 2012). Therefore, wet soil, plant and water may all represent potential sources of *P. insidiosum* infection.

We previously reported the first successful isolation of *P. insidiosum* from aquatic environmental samples by culture, including confirmation using molecular methods (Supabandhu et al., 2008). Although various other species in the genus *Pythium* have been isolated from soil (Carlile et al., 2001), whether soil maintains viable *Pythium insidiosum* remains unknown. Demonstrating the presence of *P. insidiosum* in soil is an important step towards determining the extent of the environmental niche that *P. insidiosum* occupies, and the potential risk of exposure to susceptible individuals. However, traditional culture methods are not sensitive enough to detect certain fungi in soil, due in part to the presence of high numbers of rapidly-growing soil microorganisms that confound culture-detection. In an effort to solve this problem, studies have focused on developing a sensitive and specific PCR assays in order to detect pathogenic fungi (Burgess et al., 2006; Pryce-Miller et al., 2008; Theodoro et al., 2005; Zhang et al., 2005).

In this study, we develop a nested PCR assay to detect *P. insidiosum* in soil and demonstrate its high sensitivity and specificity against a broad panel of related fungal species. We then applied this method to detect *P. insidiosum* in soil samples collected from endemic areas of pythiosis in northern Thailand in tandem with conventional culture methods. The success of our assay in using molecular methods to detect *P. insidiosum* in agricultural environments will lead to new approaches for investigating the epidemiology of this enigmatic and little-known human pathogen.

Materials and methods

Microorganism

Pythium insidiosum strain CBS119452, which was recovered from a pythiosis patient in 2001, was grown on Sabouraud's glucose agar (SGA) at 28 °C. This isolate was used as the control organism throughout the study.

Soil sampling and DNA extraction

A total of 303 soil samples were collected from 25 different irrigation sources in areas that are known to be endemic for pythiosis in northern Thailand. These areas included the provinces of Chiang Mai, Chiang Rai, Lumphun, Lumpang and Nan. Twelve soil samples (three samples/site) were collected 1 m away from the banks of waterbodies from each location. From each site, 20–30 g of soil was taken at a depth of about 1 in. from the surface. The samples were kept at room temperature and DNA extraction was performed on the date that samples were taken, or within 1–2 days. Total DNA was extracted from the soil samples using a modified conventional method (Porteous et al., 1997). Briefly, 1 ml of lysis buffer solution (100 mM Tris–HCl [pH 8.0], 100 mM Sodium ethylenediaminetetraacetic acid (Na_2 EDTA) [pH 8.0], 1.5 M NaCl) was added to 200 mg soil in a 2 ml microtube containing 0.75 g of 0.5 mm diameter glass beads. The mixture was homogenized using a Mini bead beater (Biospec, Bartlesville, USA) at full speed for 30 s, 4 times, and a final bead-beating for 5 s after the addition of 100 μl of 20% SDS solution. The mixture was incubated for 1 h at 65 °C and centrifuged at 13,000 × *g* at 4 °C for 15 min. The supernatant (600 μl) was mixed with 75 μl of 5 M potassium acetate and 250 μl of 40% polyethylene glycol 8000. The crude extract was precipitated by centrifugation after incubation for at least 1 h at −20 °C and the pellet dissolved in 900 μl of 2× CTAB, then vortexed for 1 min, and incubated at 68 °C for 15 min. An equal volume of chloroform was added, gently mixed and centrifuged. The DNA was precipitated from an aqueous solution using isopropanol, followed by a subsequent ethanol precipitation. The final DNA pellet was suspended in 450 μl of 1× TAE buffer solution, introduced into a microconcentrator column (Nanosep® 100k), and centrifuged. After washing two times with 450 μl of TAE solution, 450 μl of TAE was added and centrifuged. The DNA sample in the remaining supernatant was stored at −20 °C.

Primers and PCR conditions

Primers for the first round of the nested PCR assay comprised universal fungal primers that amplify the internal transcribed spacer (ITS) ribosomal DNA region (White et al., 1990): primer ITS1, 5′-TCCGTAGGTGAACCTGCGG-3′; ITS4: 5′-TCCTCCGCTTATTGATATGC-3′. These were followed by a second round of amplification using *P. insidiosum* – specific inner primers, PI1 and ITS2R: primer PI1, 5′-TTCGTCGAAGCGGACTGCT-3′; primer ITS2R, 5′-ATAACCAGCGTCCAGT TCG-3′.

The first-round PCR assay was performed in 25-μl volume, containing 10 mM of each primer, 12.5 μl of Taq Master Mix (Qiagen or Amersham), and 1 μl of DNA extract (approximately 10 ng of DNA template). The amplification conditions for first PCR (using primers ITS1, ITS4) were 95 °C for 5 min, 35 cycles at 95 °C for 45 s, 55 °C for 1 min, 72 °C for 2 min, and a final extension at 72 °C for 10 min. PCR was carried out on a GeneAmp PCR system 2700 thermocycler (Applied Biosystems). Subsequently, the PCR amplicons were diluted and used as a template for the second round nested PCR using the following conditions: 95 °C for 5 min, 30 cycles at 95 °C for 30 s, 60 °C for 1 min, 72 °C for 1 min, and a final extension at 72 °C for 10 min. The amplified products were analyzed by agarose gel electrophoresis, followed by ethidium bromide staining.

Sensitivity and specificity of the assay

The sensitivity of the PCR was determined by PCR assay using serially diluted DNA extracted from *Pythium insidiosum* strain CBS119452 that were spiked into an extracted *P. insidiosum*-negative soil sample. The specificity of the inner primer pair was determined by PCR assay using DNA from the following fungi, including closely-related species in the genus *Pythium*.

Sequencing of PCR products

The PCR products were cloned into pTZ57R/T vector (InsTAclone™ PCR Cloning Kit; Fermentas) and transformed into XL-1 blue *E. coli* competent cells using the manufacturer's protocol. Nucleotide sequencing was then performed using the Sanger method (1st BASE Sequencing; First BASE Laboratories Sdn Bhd, Malaysia). The sequences were analyzed by BLAST search homology of the GenBank database.

Sequencing analysis

All sequences generated in this study have been deposited in GenBank and their accession numbers are listed in Table 1. The ITS sequences of selected soil extract samples representative of each location were analysed by BLAST comparisons against GenBank sequences. Sequences were aligned using ClustalW and manually edited using MEGA4 program version 4.0 (Tamura et al., 2007). ITS sequences of *P. insidiosum* and *Pythium* sp. available from the GenBank database were also included among the alignments.

Download English Version:

https://daneshyari.com/en/article/8385743

Download Persian Version:

https://daneshyari.com/article/8385743

Daneshyari.com