

# EVALUATION OF NESTED PCR TECHNIQUE FOR DETECTION OF *PYTHIUM INSIDIOSUM* IN PATHOLOGICAL SPECIMENS FROM PATIENTS WITH SUSPECTED FUNGAL KERATITIS

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**Abstract.** Diagnosis of *Pythium* keratitis is problematic due to the difficulty in obtaining a culture report resulting in unnecessarily prolonged usage of antimicrobial medication due to misdiagnosis. This study evaluated and compared nested PCR technique with culture and immunoperoxidase staining assays of *Pythium insidiosum* in paraffin-embedded corneal tissues from patients with suspected fungal keratitis. Six of 51 pathological reports compatible with fungal infection and 6 of 48 culture-proven fungal keratitis were identified as *Pythium*. Twenty-seven specimens were PCR-positive for *Pythium insidiosum*. In comparison with fungal culture for *P. insidiosum*, PCR had 83% sensitivity and 77% specificity with fair agreement (Kappa score of 0.227,  $p = 0.001$ ). The mean age of PCR-positive is younger than PCR-negative group and there is a female preponderance in *Pythium*-infected group ( $p = 0.002$  and  $p = 0.004$ , respectively). Nineteen specimens had positive results using immunoperoxidase staining assay with fair agreement to culture method (Kappa 0.340,  $p < 0.001$ ), and 83% sensitivity, 85% specificity and 85% accuracy (95% CI: 76.7-90.7). PCR-based technique compared with culture and/or immunoperoxidase staining assay had 91.7% sensitivity, 81.8% specificity and 83% accuracy (95% CI: 74.5-89.1) with moderate agreement (Kappa 0.477,  $p < 0.001$ ). Thus nested PCR detection of *P. insidiosum* should be employed in preliminary diagnosis of *Pythium* keratitis in order to initiate proper management.

**Keywords:** *Pythium insidiosum*, fungal keratitis, nested PCR, paraffin-embedded tissue, immunoperoxidase staining assay

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## INTRODUCTION

*Pythium insidiosum*, an aquatic fungal-like organism, is commonly found in ponds and swampy areas and can infect not only animals, but also humans (Supabandhu *et al*, 2008). The first report of a

*P. insidiosum* patient with thalassemia and chronic skin infection was from Thailand (Thienprasit, 1986). This fungal-like infection mostly involves the artery causing arteritis in 59% of patients with rapid and serious clinical consequences and can lead to death (Krajaejun *et al*, 2006). *Pythium* keratitis or ocular pythiosis is the 2nd common form and is found in 33% of patients (Thienprasit *et al*, 1996). In spite of occurrence mostly in healthy patients, delayed management of *Pythium* keratitis may result in corneal perforation requiring penetrating keratoplasty in order to preserve vision (Prasertwitayakij *et al*, 2003). However, because of the unresponsiveness to most antifungal drugs, immediate excision or enucleation is crucial in severe uncontrolled infection or endophthalmitis in order to prevent progression to the brain (Murdoch and Parr, 1997).

Diagnosis of *Pythium* keratitis is problematic due to the difficulty in obtaining a culture report as there is no growth within 3 weeks resulting in unnecessarily prolonged usage of antimicrobial medication at great expense and time loss due to misdiagnosis (Virgile *et al*, 1993). *Pythium* keratitis diagnosed by pathological examinations in 10 patients during a 10 year period in 1988-1998 was mostly in farmers and resulted finally in enucleation (Kunavisarut *et al*, 2003). Thalassemia is associated with *Pythium* keratitis in only 9% of patients, less than that with *Pythium* vasculitis (Krajaejun *et al*, 2004).

DNA-based and serology methods were found to be faster than culturing in identifying *Pythium* (Vanittanakom *et al*, 2004). Similarly, polymerase chain reaction (PCR)-based DNA sequencing technique targeting internal transcribed spacer (ITS) region was a rapid and reli-

able method in 54% of the tests (Bagyalakshmi *et al*, 2008). Immunobiological diagnosis of *P. insidiosum* by blood test has also been reported (Srimuang *et al*, 1996), but sensitivity and specificity to confirm accuracy of this test were not shown.

The purpose of this study was to evaluate nested PCR technique for detection of *P. insidiosum* in pathological specimens of patients with suspected fungal keratitis compared with culture method and immunoperoxidase-staining assay.

## MATERIALS AND METHODS

### Samples

Patients' charts, pathological reports and culture results stored at the Department of Pathology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand were retrospectively reviewed in 100 pathological specimens from patients clinically suspected of fungal keratitis or an unknown cause. The study was approved by the Institutional Review Board of Faculty of Medicine Siriraj Hospital, Mahidol University, 2010-2011.

Each specimen was sliced into 20 pieces of 5 µm thickness and singly placed into a sterile Eppendorf tube containing 1.2 ml of xylene for deparaffinization. After sedimentation, an equal volume of 96% ethanol was added, the sample sedimented and supernatant removed. The process was repeated once more. After drying at 37°C, DNA was extracted using QIAamp DNA Mini Kit (Hilden, Germany) following manufacture's instructions (Wright and Manos, 1990). DNA was stored in 20 µl AE buffer at -20°C before used.

### Nested PCR detection of *P. insidiosum*

Nested PCR was conducted using CPL 6/CPR 8 as outer primers and

Table 1  
Temperature, duration and number of cycles used in nested PCR.

PCR	Primer	Denaturation		Annealing		Extension		No. of cycles
		Temp (°C)	Time (min)	Temp (°C)	Time (min)	Temp (°C)	Time (min)	
1	CPL6-CPR8	95	5	-	-	-	-	1
		95	1	68	1	72	1	30
2	YTL1-YTR1	95	1	-	-	-	-	1
		95	1	57	1	72	1	30

Table 2  
Primers used in amplification of *Pythium insidiosum* 18S rDNA.

Primer	Sequence 5' - 3'	Position corresponding to <i>P. insidiosum</i> 18s rRNA gene (accession number AF442497)	T <sub>m</sub> (°C)	% GC
CPL6	GACACAGGGAGGTAGTGACAATAAATA	434-460	76	41
CPR8	CTTGGTAAATGCTTTCGCCT	926-945	58	45
YTL1	CTTTGAGTGTGTTGCTAGGATG	646-667	64	45
YTR1	CTGGAATATGAATACCCCAAC	864-885	64	45

T<sub>m</sub>, temperature.

YTL 1/YTR 1 as inner primers as previously described (Tongsee, 2007) and the procedures are summarized in Table 1, generating an amplicon of 512 and 220 bp, respectively. DNA of *P. insidiosum* CBS 673.85 was used as reference DNA. Sequences of primers used for amplification of *P. insidiosum* 18 S rDNA, melting temperatures and amplicons are shown in Table 2.

### Immunoperoxidase staining

Immunoperoxidase staining of *P. insidiosum* was performed as previously described (Keeratijarut *et al*, 2009). In brief, a 4- $\mu$ m thick paraffin-embedded tissue was deparaffinized in xylene, washed

with ethanol and phosphate-buffered saline (PBS), pH 7.4. An aliquot of Tris-EDTA buffer (pH 9.0) (TE) was added and sample incubated at 95°C for 40 minutes, followed by 3% hydrogen peroxide in methanol for 30 minutes. After incubating in 3% horse albumin in PBS for 20 minutes, rabbit antiserum, prepared by injecting *P. insidiosum* extract, was added (1:16,000 dilution) and incubated for 1 hour at room temperature. The tissue section was washed twice with PBS before incubating with 200  $\mu$ l of the secondary antibody (undiluted mouse anti-rabbit antibody conjugated with horseradish-peroxidase; EnVision+System-Labeled Polymer-HRP, Dako, Carpinteria, CA) for

30 minutes. Then, 0.1% diaminobenzidine in Tris buffer (pH 7.4) was added and hematoxylin staining was performed before examination under a light microscope. The presence of brownish stained hyphal element indicates *P. insidiosum*, while an unstained cell indicates other fungi.

### Statistical analysis

Statistical procedures were analyzed using PASW Statistics 18 (SPSS, Chicago, IL). Mean and standard deviation are described for quantitative data, while number and percentage are reported for qualitative data. Sensitivity, specificity, accuracy and 95% confidence interval were used to evaluate the validity of PCR diagnosis of *Pythium* keratitis, compared with culture and immunoperoxidase staining methods. Kappa statistics was used to assess diagnostic agreement. Chi-square test (Pearson's, Yates' continuity correction or Fisher's exact test) for qualitative variables and unpaired *t*-test for quantitative variables were used to compare differences between the two groups as appropriate. A two-tailed *p*-value < 0.05 is considered statistically significant.

## RESULTS

There were 62 and 38 male and female patients, respectively with average age of  $52.5 \pm 15.9$  years ( $\pm$  standard deviation) with a range of 3-84 years. Mean intraocular pressure was  $22.5 \pm 10.7$  mmHg. (min 2, max 50), with 36/69 (52%) patients having intraocular pressure > 21 mmHg. Sixty-six patients presented with penetrating keratoplasty whereas enucleation and corneal biopsy were performed in 30 and 4 patients, respectively. Forty-four right and 56 left eyes were affected.

Nineteen patients had associated diseases, such as hypertension (*n* = 8), diabetes mellitus (4), heart disease (2),

HIV-AIDS (2), asthma (1), Alzheimer's (1), nasopharynx carcinoma (1), facial palsy (1), gout (1),  $\beta$ -thalassemia (1), hypothyroid (1), hemoglobin H disease (1), malnutrition (1), and systemic lupus erythematosus (1). Sixty-seven patients had a history of associated cause of corneal ulcer. Eighty-four patients had best corrected visual acuity of blindness (< 3/60).

Average size of epithelial defect of corneal ulcer was  $25.6 \pm 18.6$  mm<sup>2</sup> (range 2.4-90) and that of stromal infiltrate  $25.0 \pm 18.1$  mm<sup>2</sup> (range 2.0 - 88.2). Shapes of corneal ulcers were round (*n* = 66), oval (27), triangle (3), dumbbell (2) and geographic (2). Ninety-one patients had total depth of corneal lesion, 3 with anterior to deep stroma, 3 with anterior to mid stroma, 1 with mid-stroma, and 2 with deep stroma. Sixty-four patients had central location of corneal ulcer, 21 paracentral, 10 total cornea, and 5 peripheral. There were hypopyon in 62 patients, endothelial plaque in 16, satellite lesion in 13, descemetocoele in 12 and feathery edge in 1.

History of pathological result with fungus in 47 patients was reported, but a second review of the slides reported 51 samples with fungus (Kappa score of 0.80, *p* < 0.001).

Eight patients had positive culture with the following bacterial co-infection: *Staphylococcus* coagulase negative (*n* = 3), *Streptococcus pneumoniae* (2), *Acinetobacter* (1), *Clostridium perfringens* (1) and *Pseudomonas aeruginosa* (1). One out of 25 patients had *Acanthamoeba* growth. Forty-eight patients had positive results of fungal growth: *Fusarium* (*n* = 19), *Aspergillus* (4), *Curvularia* (2), *Candida albicans* (1), *Scedosporium apiospermum* (1), *Acremonium* (1), dematiaceous mold (6), nonsporulate septate molds (3), hyaline mold (2), non-septate mold (2), few molds (1), *Pythium*

Table 3  
Age, sex, associated diseases and culture result.

	Fungal culture, <i>n</i> (%)			<i>p</i> -value
	<i>Pythium</i> ( <i>n</i> = 6)	Other fungus ( <i>n</i> = 42)	No growth ( <i>n</i> = 52)	
Age, mean ± SD (years)	44.7 ± 11.1	52.0 ± 13.4	53.8 ± 18.1	0.397
Female	5 (83)	14 (33)	19 (36)	0.059
Associated disease	1 (17)	6 (14)	12 (23)	0.552
Ocular disease	0 (0)	1 (2)	4 (8)	0.424

Table 4  
Sensitivity, specificity, accuracy and agreement of PCR detection of *Pythium* compared with culture and immunoperoxidase staining methods.

	PCR				<i>p</i> -value
	Sensitivity	Specificity	Accuracy (95% CI)	Kappa	
Culture	83	77	77 (67.9 - 84.2)	0.227	0.001
Immunoperoxidase staining	68	83	80 (71.1 - 86.7)	0.440	0.001
Culture and staining	100	77	78 (68.9 - 85)	0.249	<0.001
Culture or staining	65	83	79 (70.2 - 85.8)	0.420	<0.001
Culture and/or staining	91.7	81.8	83 (74.5 - 89.1)	0.477	<0.001

*insidiosum* (4) and *Pythium* sp (2).

Among *Pythium* keratitis (*n* = 6), 3 showed moderate ulcer (2-6 mm), 2 with severe ulcer (> 6 mm) and 1 with mild ulcer (< 2 mm). There were 6 positive results of *Pythium* sp among the 100 suspected fungal specimens and 6/48 culture-proven fungus were found (Table 3).

#### Nested PCR detection of *P. insidiosum*

Twenty-seven out of 100 specimens had positive results for *P. insidiosum* using nested PCR (Table 4), with 17 (63%) having moderate ulcer and 10 (37%) with severe ulcer. There was 83% sensitivity, 77% specificity, and 77% accuracy (95% confidence interval (CI) : 67.9 - 84.2) for PCR detection of *Pythium* compared with fungal culture (Kappa score of 0.227,

*p* = 0.001). PCR-based detection of *Pythium* yielded more positive results in the younger age group (45.6 ± 14.5 years) (*p* = 0.002) and in females (*p* = 0.004).

#### Immunoperoxidase staining for *P. insidiosum*

Nineteen out of 100 samples were positive for *P. insidiosum* by immunoperoxidase staining method, with 83% sensitivity, 85% specificity and 85% accuracy (95% CI: 76.7-90.7) compared with culture technique (Kappa score of 0.340, *p* < 0.001), and 68% sensitivity, 83% specificity and 80% accuracy (95% CI : 71.1-86.7) compared with PCR-based method (Kappa score of 0.440, *p* < 0.001). In comparison with the pathological evaluation of reviewed slides. Kappa score was 0.368, *p* < 0.001.

## DISCUSSION

*Pythium* keratitis is a serious disease and is difficult to treat, eventually resulting in enucleation. Thus early diagnosis may lead to a more proper management to preserve vision.

This study is the first report on the sensitivity, specificity, accuracy and agreement of PCR-based method for *Pythium* detection in corneal samples compared with not only the gold standard fungal culture method but also with histodiagnosis by immunoperoxidase staining assay, resulting in 91.7% sensitivity, 81.8% specificity and 83% accuracy (95% CI: 74.5-89.1) (Kappa score of 0.477,  $p < 0.001$ ) (Table 4).

Contrary to other reports (Vanittanakom, 2004), females and patients of young age yielded more PCR positive results for *Pythium*. In order to understand this phenomenon epidemiology of *Pythium* keratitis should be thoroughly investigated with greater awareness of unresponsiveness to treatment in chronic corneal ulcers with negative culture results. Although positive culture of *Pythium* is the definitive diagnosis, this can mean a delay of 3 weeks or more, during which endophthalmitis can occur leading up to enucleation in 80% of the cases (Krajaejun *et al*, 2006). Many antifungal agents have been used and penetrating keratoplasty has been performed in *Pythium* keratitis, it still remains an uncontrollable infection (Tanhehco *et al*, 2011). Therefore, nested PCR detection of *Pythium* will have an essential role in the preliminary diagnosis as evidenced in previous reports (Badenoch *et al*, 2001; Vanittanakom *et al*, 2004; Thanathanee *et al*, 2013).

The clinical findings of *Pythium* keratitis in PCR positive group are similar to

those of the PCR negative group, with moderate severity. Despite the positive results of immunoperoxidase staining assay with high accuracy of biopsy tissues, the technique requires special staining reagent and is more expensive than the PCR technique. However, it can be employed to confirm diagnosis by requiring only a pathologist to interpret and detect the presence of *P. insidiosum*, and the immunoperoxidase staining assay will be beneficial in situations where there is no growth in culture.

The difficulty in diagnosing *P. insidiosum* is that it relies on the expertise of microbiologists and pathologists, including the knowledge regarding the location of the microorganism in pathological specimens for DNA extraction. Nevertheless, nested PCR-based technique for *Pythium* detection should be requested if clinicians suspect *Pythium* keratitis. Furthermore, as there are different incidences of *Pythium* in various geographic environments, the ability to store tissues for long periods of time, DNA diagnosis of tissue extracts will prove useful in obtaining diagnosis with high sensitivity, specificity and accuracy. The PCR-based technique can be used not only for prospective diagnosis of *Pythium* keratitis but also for retrospective diagnosis of this disease in archived samples.

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