Comparison of Nested PCR and Culture Identification of *Pythium insidiosum* in Patients with *Pythium* Keratitis

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Background: *Delayed diagnosis can lead to the development of endophthalmitis and blindness, which is difficult to manage because of the lack of an effective antimicrobial agent.*
Objective: *Comparing the nested polymerase chain reaction (PCR) technique with the standard diagnostic culture method for Pythium insidiosum.*
Material and Method: *Eighty-three patients with suspected fungal keratitis were enrolled in this observational, cross-sectional study from the Faculty of Medicine Siriraj Hospital between February 2011 and February 2014. Patient symptoms, associated diseases, duration of ulcers, precipitating causes, best-corrected visual acuity, intraocular pressure, and other clinical findings were recorded. Corneal scrapings were taken for Gram staining, bacterial and fungal cultures, staining with potassium hydroxide preparation, and DNA extraction for nested PCR. The sensitivity, specificity, accuracy, and agreement of the nested PCR analysis and culture diagnosis of P. insidiosum were compared.*
Results: *Five patients had a positive result for nested PCR amplification of P. insidiosum, while only one of these was also positive for culture growth of Pythium. Nested PCR sensitivity was 50% (95% confidence interval [95% CI] 1.3-98.7), specificity 94.7% (95% CI 86.9-98.5), and accuracy 93.5% (95% CI 85.7-97.2) with a fair agreement (kappa 0.258, p = 0.011).*
Conclusion: *Therefore, nested PCR may be an appropriate test for P. insidiosum in diagnosing Pythium keratitis with high accuracy, despite small amounts of corneal specimen.*

Keywords: *Accuracy, Nested PCR, Pythium insidiosum keratitis, Sensitivity, Specificity*

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Pythium insidiosum lives in ponds or swamps in many countries with hot climates including Thailand[1]. This fungus-like organism can infect animals such as horses, cattle, dogs, cats, and humans[2,3]. It typically invades the blood vessels as arteritis of the limb (in 59% of patients), leading to the formation of thrombi with a mortality of 40%[4]. This can also result in tissue necrosis with ischemia in several organs for instance, the skin (subcutaneous form, in 5% of patients), gastrointestinal tract, brain, disseminated involvement (3%), and the cornea as *Pythium* keratitis or corneal ulcers[4].

Delayed diagnosis can lead to the development of endophthalmitis and blindness, which is difficult to manage because of the lack of an effective antimicrobial agent[4,5]. The culture of a fungus-like organism from a corneal scraping is the standard method of diagnosis, but this is a time-consuming process that takes over four weeks to complete, and few specimens from corneal lesions are available for identification. Moreover, slow or poor growth of the culture can result in a delayed diagnosis or misdiagnosis. In such cases, keratoplasty or enucleation is necessary to prevent intraocular and systemic dissemination, and enable proper vision management[6,7].

Nested polymerase chain reaction (PCR) amplification of *P. insidiosum* from patient pathological specimens in formalin-embedded tissue has previously been shown to offer high specificity and accuracy[8]. Therefore, it is conceivable that nested PCR could offer the advantage of rapid diagnosis that could be confirmed by culture. The purpose of the present study was to compare the sensitivity, specificity, and accuracy of nested PCR with culture in the diagnosis of *P. insidiosum* infection in patients with suspected fungal keratitis.

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Material and Method

Eighty-three patients with suspected fungal keratitis lasting for more than one week were enrolled between February 2011 and February 2014. Written informed consent was obtained from all patients. This prospective, observational, cross-sectional study was approved by the Ethical Committee of the Institutional Review Board of the Faculty of Medicine Siriraj Hospital (approval number 712/2010). The medical history of patients and duration of symptoms including age, sex, associated eye diseases, and causes were recorded. Eye examinations included slit lamp biomicroscopy, the determination of uncorrected visual acuities, and measurement of intraocular pressure using a Tonopen. The size, shape, depth, location, and details of lesions were also determined.

Corneal scrapings were taken for Gram staining, potassium hydroxide (KOH) staining, bacterial culture in blood agar and chocolate agar, anaerobic culture in thioglycollate broth, *Acanthamoeba* culture in non-nutrient agar with *Escherichia coli*, and fungal culture on Sabouraud dextrose agar with or without chloramphenicol in routine laboratory. Cultures were attempted at least twice to confirm the presence or absence of growth.

Corneal specimens from all patients were inoculated into a Mycobacteria growth indicator tube (MGIT) (Becton Dickinson, Sparks, MD) for the isolation of fungus and molecular identification of *P. insidiosum* by nested PCR. Nutrient media and antibiotics (PANTA, Becton Dickinson) were added to MGITs before incubation at 25°C for five days. In the case of fungal growth, samples were examined microscopically by the teasing slide technique and staining with lactophenol cotton blue to identify the presence of septate or non-septate hyphae for later subculture. Only samples with non-septate hyphae were genetically identified by nested PCR for *P. insidiosum* using the two-tube technique[8]. All samples that did not grow were screened by nested PCR.

Nested PCR

On day 5 of incubation, 1 ml of liquid MGIT medium was transferred to a 1.5-ml sterile Eppendorf microtube, centrifuged at 12,000 rpm 037x12,000 g for five minutes in a 5417C bench-top centrifuge (Eppendorf, Hamburg, Germany). The supernatant was decanted and the pellet was washed with 10 mM Tris-HCI, 1 mM EDTA buffer, pH 8.0 (TE buffer) and collected by centrifugation at 12,000 rpm 037x12,000 g for five minutes. The supernatant was discarded, and the pellet was resuspended in 500 µl TE buffer and transferred to a 1.5-ml sterile Eppendorf microtube containing 0.4 ml siliconized glass beads (diameter, 300-500 µm).

DNA was extracted using the glass beads technique. Briefly, the tube was tightly wrapped with plastic wrap and vigorously vortexed in a Vortex Genie 2 (Scientific Industries Inc., NY) for 10 minutes at room temperature, then heated at 95 to 100°C in a heating block or water bath for 20 minutes. The supernatant was separated by centrifugation at 12,000 rpm for five minutes, then 400 µl supernatant was removed to a new sterile 1.5-ml Eppendorf microtube containing 40 µl 5 M sodium chloride, and mixed thoroughly. DNA was precipitated with cold absolute ethanol and kept at -20°C for 30 minutes. The tube was then centrifuged at 12,000 rpm for 10 minutes at 4°C in a Biofuge fresco (Heraeus, Hessen, Germany). The supernatant was discarded and the DNA pellet washed with 500 µl 70% ethanol and then centrifuged at 12,000 rpm for 10 minutes at 4°C. The supernatant was discarded and the tube was further incubated at 35°C for two hours or 65°C for 30 minutes to dry the DNA. The DNA pellet was dissolved in 20 µl TE buffer and kept at -20°C until required.

Nested PCR was carried out using the outer pair of primers CPL6, 5′-GACACAGGGAGGTAGT GACAATAAATA-3′ (corresponding to *P. insidiosum* 18S rRNA (accession number AF442497) position 434-460) and CPR8, 5′-CTTGGTAAATGCTTTC GCCT-3′ (corresponding to *P. insidiosum* position 926-945). This amplified a 512-bp template for use in the second round of PCR, which amplified a 240-bp product using primers YTL1, 5′-CTTTGAGTGTGT TGCTAGGATG-3′ (position 646-667) and YTR1, 5′-CTGGAATATGAATACCCCCAAC-3′ (position 864-885)[9].

Positive infection with *P. insidiosum* could be visualized on agarose gel electrophoresis as either a specific band at 240 bp or both a 512 bp and 240 bp product.

Main outcome measure

The nested PCR result was compared with that of *P. insidiosum* culture as the gold standard of diagnosis for sensitivity, specificity, accuracy, and agreement. Positive and negative predictive values were determined.

Statistical analysis

Data were analyzed using PASW statistics 18.0 (SPSS Inc., Chicago, IL, USA). Continuous variables

were expressed as means ± standard deviations, or medians and ranges, as appropriate. Numbers and percentages were described for categorical variables. Sensitivity, specificity, positive predictive values, negative predictive values, accuracy with 95% confidence intervals (95% CI), and data agreement were also analyzed. One-way ANOVA or the Kruskal-Wallis test were used to evaluate significant differences between keratitis groups via pair-wise comparisons using Tukey's HSD or the Dunn-Bonferroni approach, as appropriate. The Chi-square test was used to compare categorical variables among keratitis groups.

Results

Of the 83 patients recruited, three were excluded from the final analysis because of a loss of specimens. Forty-nine patients had associated causes (61.3%) (Table 1). The mean duration of symptoms was 14.9±13.4 days (median 11.5, range 1-60 days). The mean age of patients was 55.1±15.4 years (range 14-85 years), 35% of affected patients were female, and the right eye was affected in 44 eyes (55%). An intraocular pressure greater than 21 mmHg was detected in 33 of 72 patients who underwent intraocular pressure measurement (45.8%). Fifty-three patients (66.2%) had hypopyon. Sixty-six patients (82.5%) had severe corneal ulcers. Twenty-four patients (30%) had associated diseases, which hypertension was the most common (18.8%). Painful eyes, blurred vision, red eyes, and the sensation of a foreign body were common symptoms. Seventy patients (87.5%) had uncorrected visual acuities classified as blindness. Clinical characteristics of *Pythium* keratitits or corneal ulcers did not differ significantly according to fungal or bacterial or other causes (Table 1). ANOVA revealed a significant difference in age among keratitis groups (p = 0.011), while the post-hoc test found only a marginally significant difference in age between the no growth and *Pythium* groups (p = 0.011) and *Pythium* and bacteria groups (p = 0.042). The Kruskal-Wallis test showed that the duration of symptoms among keratitis groups was significant (p = 0.006), though post-hoc (Dunn-Bonferroni) tests revealed a significant difference only between two keratitis groups ([no growth] vs. [bacterial]; p = 0.018). There was no evidence of sex difference in either kind of keratitis (p = 0.069).

Positive bacterial and fungal cultures were reported in 18 (22.5%) and 12 (15%) of cases, respectively (Tables 2, 3). The *Pseudomonas* was the most common type of bacterial growth, while *Fusarium* was the most commonly identified fungus. Five of 77 patients (6.5%) had a positive nested PCR result for *P. insidiosum*, of which only one was also positive for culture (Table 4). Two of 77 patients (2.6%) had a positive culture result. One female patient had history of contact lens wearer, but another patient had ocular trauma with insect.

Nested PCR was compared with culture using a 2x2 statistical analysis. The sensitivity of

Table 1. Clinical characteristics of patients infected with *Pythium* keratitis

	n (%)						
	Pythium insidiosum (n = 2)	Other fungus (n = 22)	Bacteria (n = 21)	Bacteria + fungus (n = 1)	No growth (n = 34)	Total (n = 80)	*p*-value
Mean age ± SD (years)	25.0±15.6	52.2±11.7	54.3±16.1	54	59.2±15.4	55.1±15.4	0.011[a]
Mean duration of symptom ± SD (days)	36.0±8.5	12.1±8.2	10.6±15.1	11	18.2±14.0	14.9±13.4	0.006[a]
Females	2 (100)	6 (27.3)	10 (47.6)	1 (100)	9 (26.5)	28 (35.0)	0.069
Associated causes	2 (100)	15 (68.2)	13 (61.9)	0 (0)	19 (55.9)	49 (61.3)	0.447
Severe ulcers	2 (100)	20 (90.9)	17 (81.0)	1 (100)	26 (76.5)	66 (82.5)	0.626
Blindness	2 (100)	20 (90.9)	18 (85.7)	1 (100)	29 (85.3)	70 (87.5)	0.928
Depth >75% of corneal thickness	2 (100)	12 (54.5)	15 (71.4)	1 (100)	27 (79.4)	57 (71.3)	0.257
Central location	2 (100)	12 (54.5)	10 (47.6)	1 (100)	21 (61.8)	46 (57.5)	0.495
Round lesion	1 (50)	12 (54.5)	12 (57.1)	0 (0)	15 (44.1)	40 (50.0)	0.721
IOP >21 mmHg	0 (0)	11/21 (52.4)	12/19 (63.2)	N/A	10/30 (33.3)	33/72 (45.8)	0.100[a]
PCR for *Pythium*	1 (50)	0 (0)	2/20 (10.0)	0 (0)	2/32 (6.3)	5/77 (6.5)	0.083

IOP = intraocular pressure; PCR = polymerase chain reaction; N/A = not applicable
[a] Analysis excluded bacterial + fungus group

Table 2. Bacteria and fungi identified on three different occasions of culture on blood agar

	n (sampling occasion)		
	First	Second	Third
Positive for bacterial culture (all)	18	6	1
Pseudomonas	12	2	-
Staphylococcus	3	2	-
Propionibacterium acnes	1	-	-
Acinetobacter baumannii	-	-	1
Haemophilus influenzae	1	-	-
Non-fermenting Gram negative rod	-	1	-
Streptococcus alpha-hemolytic	1	-	-
Bacillus	-	1	-
Aspergillus	5	-	1
Fusarium	4	5	-
Molds	3	-	-
Pythium insidiosum	1	1	-
Scedosporium	1	-	-
Lasiodiplodia	1	-	-

Table 3. Fungi identified on three different occasions of culture on Sabouraud agar

	n (sampling occasion)					
	First		Second		Third	
	sC	cC	sC	cC	sC	cC
Positive for fungal culture (all)	12	11	7	7	2	2
Fusarium	6	6	5	5	1	1
Aspergillus	3	3	1	1	1	1
Non-sporulating septate mold	-	-	-	-	-	-
Scedosporium	1	-	-	-	-	-
Curvularia	1	1	-	-	-	-
Pythium	-	-	1	1	-	-
Lasiodiplodia	1	1	-	-	-	-

sC = without chloramphenicol; cC = with chloramphenicol

Table 4. Comparison of nested PCR and culture for *Pythium*

Nested PCR result for *Pythium*	Culture result for *Pythium*		
	Negative	Positive	Total
Negative	71	1	72
Positive	4	1	5
Total	75	2	77

nested PCR for *Pythium* was 50% (95% CI 1.3-98.7), specificity 94.7% (95% CI 86.9-98.5), and accuracy 93.5% (95% CI 85.7-97.2), with a fair agreement (Kappa value 0.258, $p = 0.011$) with analysis by culture (Table 4). Positive and negative predictive values were 20% (95% CI 0.5-71.6) and 98.6% (95% CI 92.5-100), respectively.

Discussion

Pythium keratitis is mostly associated with trauma in young adults in their mid-20s, rather than bacterial or fungal corneal ulcers in adults older than 50 years, especially in female patients[8], although it can also be associated with any cause. The authors report the development of keratitis in a female patient from a contact lens but in the absence of contaminated water, while another affected patient had an ocular trauma from an insect. However, the molecular detection of *P. insidiosum* from the soil has also been reported[10]. Therefore, the immune status or other associated factors may encounter *Pythium* keratitis in these patients.

P. insidiosum can grow on different types of agar, and a positive result for bacterial culture can reflect a mixed co-infection. In the present study, the authors obtained a positive nested PCR result for *Pythium* in the absence of culture growth. The authors speculate that because the first corneal scraping is used for bacterial culture analysis before other cultures, later scrapings may contain fewer organisms, resulting in a negative fungal culture result. There are no known reports of the nested PCR two-tube technique in humans. However, Botton et al reported the use of nested PCR for *P. insidiosum* diagnosis in animal infections[11]. The possibility of *P. insidiosum* infection should be considered in cases of suspected *Pythium* keratitis with high rates of morbidity and mortality but the absence of culture growth.

In the present study, nested PCR for *P. insidiosum* was shown to have a high accuracy and specificity, and to achieve a more rapid result than the standard culture technique, as shown previously[8]. However, our investigation had a number of limitations including its small sample size, technical difficulties of the method including multiple steps for investigative PCR, the time delay of at least one week to obtain culture growth, and the fact that PCR products were not stored for confirmation by sequencing. Additionally, different extraction methods of *P. insidiosum* genomic DNA have been shown to affect molecular applications, especially PCR or genetic studies[12]. Further studies should therefore aim to develop real-time automated PCR based on a small amount of tissue for rapid detection of infection. Moreover, determination of

the lowest detection limits of serially diluted DNA from *P. insidiosum* could have enabled nested PCR sensitivity to be confirmed.

Conclusion

Nested PCR appears to be a useful rapid test for *P. insidiosum* diagnosis and can be used on small amounts of specimen.

What is already known on this topic?

An alternative more rapid diagnostic technique is necessary. Nested PCR amplification of *P. insidiosum* compared with culture technique was confirmed to be highly specific and accurate.

What this study adds?

The nested PCR technique represents a rapid means of accurately identifying *P. insidiosum* infection with small amounts of corneal sample, this has important implications for clinicians involved in the decision for surgery in patients with *Pythium* keratitis.

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Potential conflicts of interest

None.

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การเปรียบเทียบการวิเคราะห์เชื้อทางโมเลกุล (PCR) และการเพาะเชื้อ Pythium insidiosum ในผู้ป่วยกระจกตาอักเสบติดเชื้อ Pythium

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ภูมิหลัง: *การวินิจฉัยโรคกระจกตาอักเสบติดเชื้อ Pythium ที่ล่าช้าซึ่งยากต่อการดูแลรักษา เพราะว่าไม่มียาต้านจุลชีพที่ได้ผล จึงนำไปสู่ลูกตาอักเสบติดเชื้อและตาบอด*

วัตถุประสงค์: *เป็นการศึกษาเปรียบเทียบ การวิเคราะห์เชื้อทางโมเลกุล และการเพาะเชื้อ Pythium insidiosum*

วัสดุและวิธีการ: *ผู้ป่วยโรคกระจกตาอักเสบสงสัยติดเชื้อราจำนวน 83 ราย เข้าร่วมการศึกษาไปข้างหน้าแบบสังเกตอาการ และแบบตัดขวางจากคณะแพทยศาสตร์ศิริราชพยาบาล ในช่วงเวลาเดือนกุมภาพันธ์ พ.ศ. 2554 ถึง พ.ศ. 2557 ได้รับการบันทึกอาการโรคร่วม ระยะเวลาที่มีแผลกระจกตา สาเหตุนำ ตรวจวัดระดับการมองเห็นที่แก้ไขดีที่สุด วัดความดันตา และอาการแสดงทางคลินิก ทำการขูดกระจกตา เพื่อส่งตรวจย้อมสีกรัม เพาะเชื้อแบคทีเรีย เพาะเชื้อรา ย้อมสีโพแทสเซียมไฮดรอกไซด์ และสกัดดีเอ็นเอ เพื่อวิเคราะห์เชื้อทางโมเลกุล ซึ่งนำผลมาเปรียบเทียบหาความไว ความจำเพาะ ความแม่นยำ และความสอดคล้องกับวิธีการเพาะเชื้อ*

ผลการศึกษา: *พบผลบวกจากการตรวจเชื้อทางโมเลกุล 5 ราย ซึ่งมีเพียง 1 ราย ที่ได้ผลบวกจากการเพาะเชื้อ Pythium ความไวของวิธีตรวจเชื้อทางโมเลกุลพบ ร้อยละ 50 (ช่วงความเชื่อมั่น ร้อยละ 95 เท่ากับ 1.3 ถึง 98.7) ความจำเพาะ ร้อยละ 94.7 (ช่วงความเชื่อมั่น ร้อยละ 95 เท่ากับ 86.9 ถึง 98.5) และความแม่นยำ ร้อยละ 93.5 (ช่วงความเชื่อมั่น ร้อยละ 95 เท่ากับ 85.7 ถึง 97.2) โดยมีค่าความสอดคล้องพอใช้ (แคปปา 0.258 ค่าพีเท่ากับ 0.011)*

สรุป: *ดังนั้นการตรวจวิเคราะห์หาเชื้อทางโมเลกุล อาจเป็นวิธีที่เหมาะสมสำหรับการวินิจฉัยโรคกระจกตาอักเสบติดเชื้อ Pythium ที่มีความแม่นยำสูง ถึงแม้ว่าได้ตัวอย่างเนื้อเยื่อกระจกตาจำนวนน้อย*