A DNA dot hybridization model for molecular diagnosis of parasitic keratitis

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Purpose: Developing a DNA dot hybridization model for diagnosing parasitic keratitis.
Methods: Newly designed oligonucleotide probes for detecting *Acanthamoeba* and microsporidia were tested with target reference strains of *Acanthamoeba* (n = 20) and microsporidia (n = 3), and non-target microorganisms, including bacteria (n = 20) and fungi (n = 20). These probes, which had passed the preliminary tests, were then assembled as a parasite dot hybridization (PDH) model for assessing 33 clinical samples from patients with clinically suspected *Acanthamoeba* and microsporidia keratitis, including eight positives for *Acanthamoeba*, 13 positives for microsporidia, and 12 negatives for both pathogens.
Results: Two probes for detecting *Acanthamoeba* and two for detecting microsporidia passed the tests using target and non-target strains and then were assembled in the PDH model. For clinical samples, one *Acanthamoeba*-positive sample (proved with pathology) was falsely negative according to the PDH assay. The sensitivity and specificity of the PDH assay for diagnosing *Acanthamoeba* keratitis were 87.5% and 100%, respectively, while the sensitivity and specificity for diagnosing microsporidia keratitis were 100%. The infectious agent of all clinical samples of microsporidia keratitis was identified as *Vittaforma corneae* with DNA sequencing, while those of *Acanthamoeba* keratitis were caused by four species of *Acanthamoeba*, with *Acanthamoeba castellanii* found in four samples (50%, 4/8).
Conclusions: The PDH model has the potential to be a molecular assay for diagnosing *Acanthamoeba* and microsporidia keratitis. However, a prospective clinical study might be needed before the model is adopted in routine clinical practice.

Parasitic keratitis, primarily caused by *Acanthamoeba* and microsporidia, is largely underreported [1]. *Acanthamoeba* keratitis (AK) and microsporidia keratitis (MK) may be easily overlooked because they usually present with non-specific symptoms masquerading as viral or noninfectious keratitis [2,3]. Early AK or MK presenting coarse punctate epithelial keratitis can be misdiagnosed as herpetic or adenoviral keratitis or toxic keratitis, whereas late forms of AK or MK that present necrotizing stromal keratitis are easily confused with bacterial or fungal keratitis. Despite the recent outbreaks of AK [4-6] and MK [3,7,8], the two diseases remain rare compared to other forms of microbial keratitis, and this rarity may lead to misdiagnosis by most eye-care practitioners [3,9]. Misdiagnosis and treatment with topical corticosteroid for AK and MK may cause medically refractory stromal keratitis leading to disastrous ocular complications and permanent visual loss.

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Routine microbiological examination focusing on bacterial and fungal keratitis can result in underdiagnoses of AK and MK. Culturing in special media is the conventional standard method for diagnosing AK, but the sensitivity can be less than 50% [10], and the incubation time is long (3–7 days) [11]. Although the taxonomic affiliation of microsporidia is closely related to that of fungi [3], microsporidia have evolved as obligate intracellular parasites that require special cell culture systems for isolation. Direct microscopic examination enables rapid diagnosis of AK [2] and MK [3], but the technique is not sensitive enough for diagnosing light infections, owing to the requirements for large corneal scrapes and expertise in ocular microbiology. Therefore, some researchers have proposed different diagnostic tests for rapid and sensitive detection of the two types of parasitic keratitis [12-15].

DNA-based molecular techniques are useful for diagnosing infections caused by *Acanthamoeba* and microsporidia [12,15-17]. In previous studies, we developed different dot hybridization models to resolve different clinical scenarios, which were found to be sensitive and specific for diagnosing bacterial keratitis [18] and fungal keratitis [19], and for differentiating AK from herpes keratitis [20]. Therefore, the aim of

TABLE 1. TARGET AND NON-TARGET MICROORGANISMS USED FOR TESTING CANDIDATE OLIGONUCLEOTIDE PROBES.

Microorganism	Species and strain no.[a]
Target	
Acanthamoeba (n=20)	*Acanthamoeba castellanii* ATCC 30,010, ATCC 50,370, ATCC 50,374
	Acanthamoeba culbertsoni ATCC 30,171
	Acanthamoeba griffini ATCC 30,731, ATCC 50,702
	Acanthamoeba hatchetti ATCC 30,730, ATCC 50,672
	Acanthamoeba jacobsi ATCC 30,732
	Acanthamoeba lugdunensis ATCC 50,240
	Acanthamoeba mauritaniensis ATCC 50,676
	Acanthamoeba palestinensis ATCC 30,870, ATCC 50,708
	Acanthamoeba polyphaga ATCC 30,461, ATCC 30,487, ATCC 30,873
	Acanthamoeba pustulosa ATCC 50,252
	Acanthamoeba quina ATCC 50,241
	Acanthamoeba rhysodes ATCC 30,973, ATCC 50,368
Microsporidia (n=3)	*Encephalitozoon cuniculi* ATCC 50,789
	Encephalitozoon hellem ATCC 50,504
	Encephalitozoon intestinalis ATCC 50,651
Non-target	
Bacteria (n=20)	*Escherichia coli* BCRC 13,095, BCRC 15,481
	Klebsiella pneumoniae BCRC 11,644, CCUG 15,938
	Mycobacterium chelonae ATCC 35,752, CCUG 37,827
	Mycobacterium fortuitum ATCC 6841, ATCC 19,542
	Nocardia farcinica BCRC 13,364, BCRC 13,380
	Pseudomonas aeruginosa ATCC 27,853, BCRC 10,944
	Serratia marcescens BCRC 10,768, BCRC 10,948
	Staphylococcus aureus BCRC 10,780, BCRC 14,957
	Staphylococcus epidermidis BCRC 14,976, BCRC 14,988
	Streptococcus pneumoniae BCRC 10,794, BCRC 14,733
Fungi (n=20)	*Alternaria alternata* BCRC 32,888, CBS 109,455
	Aspergillus flavus BCRC 30,006, BCRC 30,009
	Aspergillus fumigatus BCRC 30,502, BCRC 32,120
	Candida albicans BCRC 20,511, BCRC 20,512
	Candida parapsilosis BCRC 20,515, BCRC 21,253
	Curvularia pallescens CBS 156.35, CBS 102,694
	Curvularia senegalensis CBS 149.71, CBS 102,171
	Fusarium oxysporum ATCC 26,225, CBS 798.95
	Fusarium solani BCRC 32,446, BCRC 32,448
	Penicillium lilacinum BCRC 31,616, CBS 100,229

[a]ATCC, American Type Culture Collection, Manassas, Va., USA; BCRC: Bioresources Collection and Research Center, Hsinchu, Taiwan; CBS, Centraalbureau voor Schimmelcultures, Utrech, The Netherlands; CCUG, Culture Collection, University of Göteborg, Sweden.

the present study was to develop a parasite dot hybridization (PDH) model as an alternative strategy for diagnosing AK and MK.

METHODS

Reference strains and clinical isolates: To assess the candidate oligonucleotide probes for detecting *Acanthamoeba* and microsporidia, 20 reference strains (12 species) of *Acanthamoeba* and three strains (three species) of microsporidia were used as the target strains for the sensitivity test, while 20 reference strains of bacteria (ten species) and 20 strains

(ten species) of fungi were used as non-target strains for the specificity test (Table 1). The probes, which had passed the preliminary tests, were then assembled in the PDH model (Table 2, Figure 1) for further clinical assessments.

Clinical specimens: A scraping procedure for corneal debridement was performed for patients with clinically suspected *Acanthamoeba* and microsporidia keratitis using a #15 sterilized knife under biomicroscopy. One portion of the scrape was sent to the laboratory for standard microbiological analyses, including direct microscopy (Gram stain and acid fast stain) [3,19] and culture (blood agar and *Escherichia coli*

Target microorganism	**Probe code[a]**	**Sequence (5' to 3')**	**Length (nucleotide)**	**GC (%)**	T_m[b] (°C)	**Location[c]**	**GenBank accession number**
Acanthamoeba	AC1	CTGCCACCGAATACATTAGCATGGtttttttttt[d]	24	50.0	59.3	1145–1168	KT185626
	AC2	GATTAACTTCTGCGAAAGCATCTtttttttttt	23	39.1	51.7	1302–1324	KT185626
Microsporidia	MS1	GATGAAGGACGAAGGCTGGAGtttttttttt	21	57.1	55.2	594–614	XR552277
	MS2	TCTGGGGATAGTATGCTCGCCAAGtttttttttt	23	52.2	57.2	712–734	XR552277

TABLE 2. THE OLIGONUCLEOTIDE PROBES USED IN THE PDH MODEL.

[a]Oligonucleotide probes are positioned on the PDH model as indicated in Figure 1. [b]T_m, melting temperature. [c]The corresponding locus of the probe to the specified GenBank accession number [d]Several bases of thymine were added to the 5' end of the probe to increase hybridization signal.

enriched non-nutritional agar for cultivation of amebae). In addition, PCR was performed for detection of *Acanthamoeba* and microsporidia [12,21]. The remaining corneal scrape on a knife was put into a 1.5-ml sterile Eppendorf tube containing 1 ml saline and stored at −20 °C before DNA extraction. Corneal biopsy for pathological examination was performed only for the patient refractory to medical treatment. For evaluation of the clinical samples with the PDH model, 33 corneal scrapes from patients with clinically suspected *Acanthamoeba* and microsporidia keratitis were consecutively collected from

July 25, 2012, to November 25, 2015, with approval from the Institutional Review Board (IRB)/the Committee of Medical Ethics and Human Experiments of National Cheng Kung University Hospital. All procedures adhered to the Declaration of Helsinki and the ARVO statement on human subjects. Among the 33 consecutively collected samples, eight were AK positive as diagnosed with direct microscopy, culture, PCR, or pathology, and 13 were MK positive as diagnosed with direct microscopy or PCR (Table 3). Control negatives included 12 scrapes in which AK and MK were excluded with
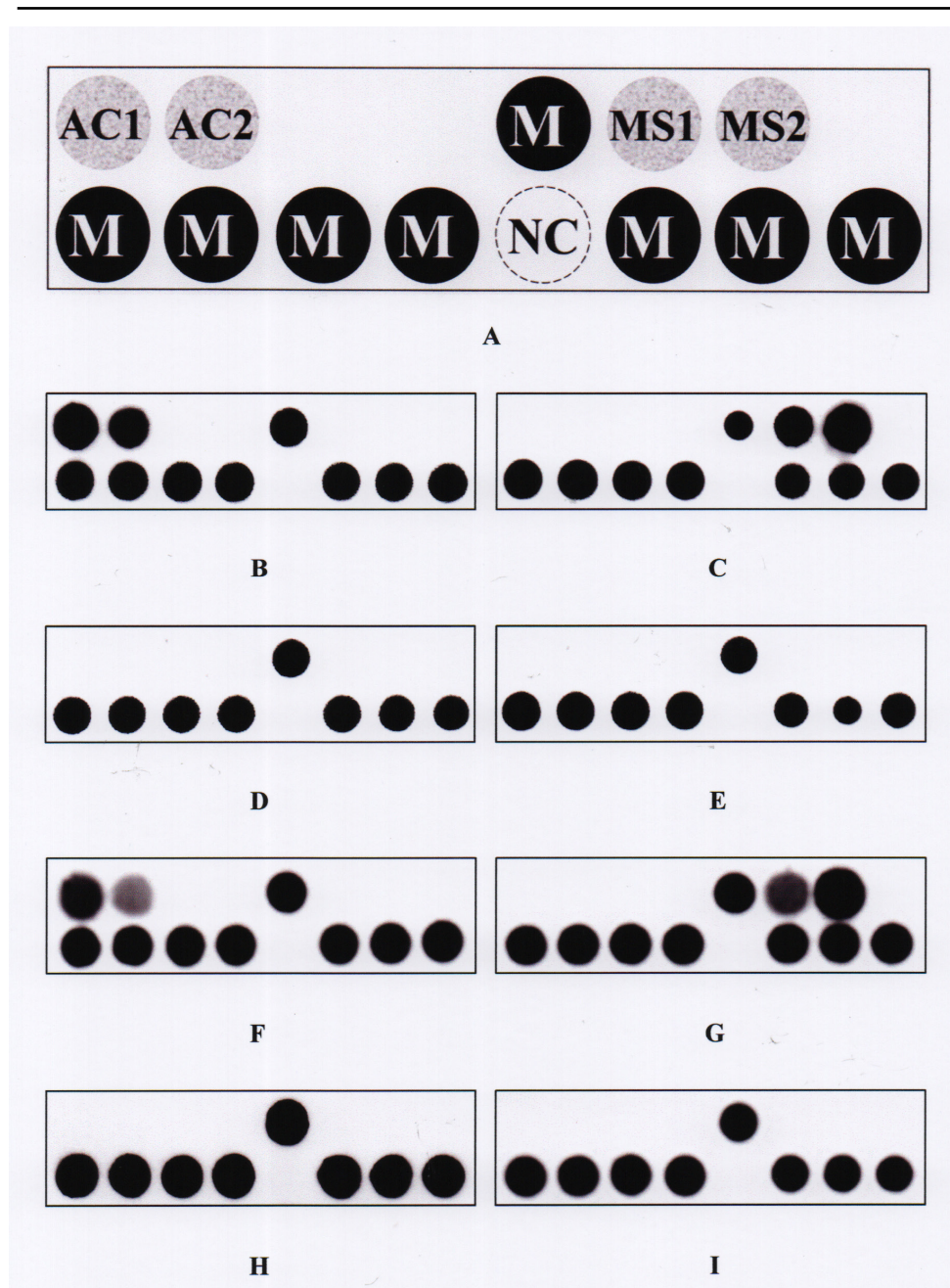

Figure 1. The PDH model. **A**: Layout of oligonucleotide probes on the model (0.8 × 0.2 cm). The probes "AC1" and "AC2" were used to identify *Acanthamoeba* spp. The probes "MS1" and "MS2" were used to identify microsporidia. The dot "NC" is a negative control (tracing dye only). The probe "M" is a position marker probe, i.e., an irrelevant digoxigenin-labeled oligonucleotide probe (5′-digoxigenin-GCA TAT CAA TAA GCG GAG GA-3′). All probe sequences are listed in Table 2. **B−E**: Representative hybridization patterns for *Acanthamoeba castellanii* ATCC 30,010, *Encephalitozoon cuniculi* ATCC 50,789, *Pseudomonas aeruginosa* BCRC 10,944, and *Fusarium solani* BCRC 32,446, respectively. **F−I**: Hybridization patterns for the represented clinical samples that were positive for *Acanthamoeba* (sample no. 1e), positive for microsporidia (sample no. 1a), false negative for *Acanthamoeba* (sample no. 1f), and true negative (sample no. 1g).

standard microbiological analyses and PCR. None of the 12 scrapes had reports of pathological examination.

DNA extraction and duplex PCR: The thawed corneal scrape in normal saline was transferred to a 1.5-ml Eppendorf tube and centrifuged at 13,200 ×*g* in a microfuge for 10 min. DNA in the precipitate was extracted using a commercial kit (DNeasy Blood & Tissue Kit, Qiagen, Valencia, CA). The extracted DNA was amplified with a duplex PCR using two pairs of primers: One pair was used to amplify the 18S rRNA gene of *Acanthamoeba* (*JDP1*, 5′-digoxigenin-GGC CCA GAT CGT TTA CCG TGA A-3′; *JDP2,* 5′-digoxigenin-TCT CAC AAG CTG CTA GGG GAGTCA-3′) [21], and the other pair was used to amplify the small subunit rRNA gene of microsporidia (*V1*, 5′-digoxigenin-CAC CAG GTT GAT TCT GCC TGA C-3′ [22], and a primer Mco807R, 5′-digoxigenin-CGC GTT GAG TCA AAT TAA G-3′ newly designed in this study). Each primer was labeled with a digoxigenin molecule at the 5′ end. The PCR mixture (25 μl) consisted of 2.5 μl template DNA, 0.2 μM each primer, and other necessary reagents from a PCR kit (KAPA2G Fast HotStart ReadyMix; Kapa Biosystems, Boston, MA). The cycling conditions were as follows: initial denaturation (95 °C, 3 min), ten cycles of denaturation (95 °C, 15 s) and annealing (60 °C, 50 s), and 36 cycles of denaturation (95 °C, 15 s), annealing (55 °C, 30 s), and extension (72 °C, 20 s). Positive controls were performed in each run by using template DNAs of *Acanthamoeba castellanii* ATCC 30,010 and *Encephalitozoon cuniculi* ATCC 50,789 (a microsporidia strain), respectively. A negative control was performed in each run by replacing the template DNA with sterile water.

Immobilization of Acanthamoeba- and microsporidia-specific oligonucleotide probes on a nylon membrane: The universal *Acanthamoeba* probes were designed from a conserved sequence in the 18S rRNA gene, while the universal microsporidia probes were designed from a conserved sequence in the small subunit rRNA gene (Table 2). The procedure for the immobilization of oligonucleotide probes on a nylon membrane is described elsewhere [23]. In brief, each probe in the PDH model (Figure 1A) was diluted 1:1 (final concentration, 10 μM) with a tracking dye solution and spotted on a positively charged nylon membrane (Roche, Mannheim, Germany) using a pin (400 μm in diameter) and a spotter (SR-A300; EZlife Technology, Taipei, Taiwan) to form an array (0.8 × 0.2 cm). A digoxigenin-labeled irrelevant oligonucleotide probe (code M, 5′-digoxigenin-GCA TAT CAA TAA GCG GAG GA-3′) was used as a position marker. The dot NC was a negative control (tracking dye only). Once all the probes had been spotted, the membrane was exposed to a shortwave ultraviolet (UV) light (Stratalinker 1800;

Stratagene, La Jolla, CA) for 30 s to fix the probes on the membrane.

Experimental procedures of the PDH model: A 10-μl aliquot of the PCR product was used for the PDH model. The procedures for prehybridization, hybridization, and color development have been described elsewhere [23]. In brief, the PDH model was prehybridized at room temperature for 1 h with 1 ml of hybridization solution (5×saline sodium citrate (SSC) [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 1% [wt/vol] blocking reagent, 0.1% N-laurylsarcosine, and 0.02% sodium dodecyl sulfate). Hybridization was conducted at 55 °C for 90 min. After removing the nonhybridized PCR products and blocking solution, alkaline phosphatase-conjugated anti-digoxigenin antibodies (Fab fragments; Roche, Mannheim, Germany) and phosphatase substrates (nitroblue tetrazolium chloride-5-bromo-4-chloro-3-indolylphosphate; Roche, Mannheim, Germany) were used for color development. *Acanthamoeba* was identified if at least one of the two probes (codes AC1 and AC2) was hybridized, and microsporidia was identified if at least one of the two probes (codes MS1 and MS2) was hybridized. Images of hybridized arrays were captured with a scanner (Perfection™ V600 Photo; Epson, Nagano, Japan).

Detection limits of the PDH model: The detection limits of the PDH model for *Acanthamoeba* and microsporidia were determined by testing tenfold serial dilutions of the prequantified DNA samples of *A. castellanii* ATCC 30,010 and *E. cuniculi* ATCC 50,789, respectively. For a sample that was positive for *Acanthamoeba* or microsporidia, the purified DNA was amplified with the respective PCR for *Acanthamoeba* and microsporidia, the amplicon was sequenced, and the determined sequence was used to search for homologous sequences of the infectious agents in GenBank using the BLASTN program.

Statistical analysis: Using the results obtained with standard microbiological methods, PCR, and/or pathological examination as the reference method, the performance indices, including sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV), for diagnosis of AK and MK were calculated. The 95% confidence intervals for the performance indices were estimated with an online calculator (Causascientia).

RESULTS

Assessment of the candidate oligonucleotide probes with target and non-target strains: All target strains of *Acanthamoeba* (n = 20) and microsporidia (n = 3) listed in Table 1 were correctly identified by the two *Acanthamoeba* probes (AC1 and AC2) and the two microsporidia probes (MS1 and MS2),

TABLE 3. POSITIVE CLINICAL SAMPLES DETERMINED BY THE STANDARD MICROBIOLOGICAL METHODS, PCR, AND THE PDH MODEL, AND IDENTIFICATION OF THE INFECTIOUS AGENTS BY DNA SEQUENCING.

Samples no.	Microscopy		Culture	Pathology	PCR[a]	PDH model	Species of the infectious microorganism[b]	Sequence similarity, %
	GS[c]	AFS[d]						
Acanthamoeba (+)								
1e	−	−	+	ND[e]	+	+	*Acanthamoeba quina*	99.3
1f	−	−	−	+	−	−	NA[f]	NA
1h	−	−	−	+	+	+	*Acanthamoeba polyphaga*	99.3
2a	−	−	+	ND	+	+	*Acanthamoeba* sp.	92.5
2d	−	−	+	ND	+	+	*Acanthamoeba castellanii*	100
2g	−	−	+	ND	+	+	*Acanthamoeba castellanii*	98.8
4g	−	−	+	ND	+	+	*Acanthamoeba castellanii*	98.8
4h	−	−	+	ND	+	+	*Acanthamoeba castellanii*	98.6
Microsporidia (+)								
1a	−	−	−	ND	+	+	*Vittaforma corneae*	99.2
1b	+	+	−	ND	+	+	*Vittaforma corneae*	100
1c	+	−	−	ND	+	+	*Vittaforma corneae*	100
1d	+	−	*Streptococcus pneumoniae*	ND	+	+	*Vittaforma corneae*	99.0
2f	−	−	−	ND	+	+	*Vittaforma corneae*	99.3
2h	−	−	−	ND	+	+	*Vittaforma corneae*	99.5
2j	+	−	−	ND	+	+	*Vittaforma corneae*	99.9
3a	ND	+	−	ND	+	+	*Vittaforma corneae*	99.9
4b	+	−	−	ND	+	+	*Vittaforma corneae*	99.9
4c	+	−	−	ND	+	+	*Vittaforma corneae*	99.9
4d	+	−	−	ND	+	+	*Vittaforma corneae*	97.0
4e	−	−	−	ND	+	+	*Vittaforma corneae*	99.6
4l	+	+	−	ND	+	+	*Vittaforma corneae*	99.7

[a]PCR, polymerase chain reaction. [b]The species names of the infectious agents were determined by gene sequencing. [c]GS, Gram stain. [d]AFS, acid fast stain. [e]ND, not done. [f]NA, not available.

respectively. No PCR product was amplified from any microorganism of non-target bacteria (n = 20) and fungi (n = 20; Table 1), and thus, no cross-hybridization of the four probes was found. In addition, all target strains of *Acanthamoeba* did not cross-hybridize with the microsporidia probes, and vice versa. The four candidate probes were then assembled in the PDH model for the following assessments with clinical specimens. The representative hybridization patterns of the PDH model are shown in Figure 1.

Detection limits of the PDH model: The serial tenfold diluted DNA samples of *A. castellanii* ATCC 30,010 and *E. cuniculi* ATCC 50,789 were assessed with the PDH model. The detection limits for both pathogens were 0.25 ng DNA per test. However, both detection limits decreased to 2.5 pg DNA if a single-plex PCR was used for *A. castellanii* and *E. cuniculi*, respectively.

Species of Acanthamoeba and microsporidia causing keratitis in clinical samples: The species causing AK (n = 7) and MK (n = 13) were further determined with DNA sequencing of the PCR amplicons followed by BLASTN search in GenBank. The microorganism in four (no. 2d, 2g, 4g, and 4h) of the seven AK positive samples was identified as *Acanthamoeba castellanii*, and the remaining three samples were identified as *A. quina* (no. 1e), *A. polyphaga* (no. 1h), and *Acanthamoeba* sp. (no. 2a), respectively (Table 3). Microsporidia in all 13 cases of MK was identified as *Vittaforma corneae* (Table 3).

Performance of the PDH model for diagnosing AK and MK: The PDH model was then used to analyze 33 clinical samples. For AK diagnosis, 32 concordant (seven positives and 25 negatives) and one discordant (negative according to the PDH model but positive according to the standard microbiological methods) results were obtained. One sample (sample no. 1d) had a positive bacterial culture (*Streptococcus pneumoniae*), but the presence of a streptococcal microorganism in this sample did not influence the diagnosis of MK by the PDH model. For diagnosis of MK, all 33 samples produced concordant results (13 positives and 20 negatives). The sensitivity, specificity, PPV, and NPV of the PDH model for diagnosis of AK were 87.5%, 100%, 100%, and 96.2%, respectively, while the respective values for MK diagnosis were all 100% (Table 4). If AK and MK were considered together, the respective performance values of the PDH model were 95.2%, 100%, 100%, and 92.3%.

DISCUSSION

This is the first study to describe a molecular test for diagnosing AK and MK simultaneously. The PDH model, based on specific oligonucleotide probes targeting the 18S rRNA gene of *Acanthamoeba* and the small subunit rRNA gene of

microsporidia, had high sensitivity and specificity (Table 4). This model requires minimal instrumentation and can be completed within one working day. Although a duplex PCR was performed in this study, a single-plex PCR targeting either *Acanthamoeba* or microsporidia can be used on demand. The detection limits (2.5 pg/assay) were 100 times lower if single-plex PCR was adopted.

The PDH model produced one false negative (sample no. 1f) for the diagnosis of AK (Table 3); this might be caused by a low cell number of amebic cells present in the specimen or even no amebic cells sampled due to a wrong sampling locus [15]. In addition, this sample (no. 1f) was positive only with pathology; the sample was negative according to the standard microbiological analyses and PCR. Therefore, a deeper infection was highly suspected, and this might result in a sampling failure. The detection rates of direct microscopy for AK were highly variable [24,25]. No sample was found to have amebic cells by Gram stain in this study (Table 3). In general, a relatively large tissue sample and expertise in ocular microbiology are required for direct microscopy.

In a previous study, we developed a pair of PCR primers and an oligonucleotide probe to detect *Acanthamoeba* in clinical samples [20]. However, the two primers had low efficiency when an additional pair of primers for microsporidia was included in the duplex PCR described in this study. This might be caused by an interaction (such as dimer formation) between the primers used to amplify *Acanthamoeba* and microsporidia. Therefore, the primers described by Schroeder et al. [21] were used in the duplex PCR, and two new probes were designed for the diagnosis of *Acanthamoeba*. The sensitivity (87.5%) of the array for *Acanthamoeba* detection was slightly lower than that (93.3%) of the previous study [20].

Compared to the results of Joseph et al. [12] (PCR using pan-microsporidian primers had a sensitivity of >83% and a specificity of 92%), the current PDH model displayed a better performance for MK diagnosis (Table 4). In addition, this PDH model being able to differentiate AK from MK might have a benefit under confusing or atypical presentations [7]. *Streptococcus pneumoniae* was isolated from one (sample no. 1d) of the microsporidia-positive samples (Table 3); the result indicated a complication of polymicrobial keratitis for this patient with MK.

The amplicons of all MK-positive samples were successfully sequenced, and a single microorganism (*Vittaforma corneae*) was identified (Table 3); the results were in agreement with those of previous studies [7,8,16]. However, at least three species of *Acanthamoeba* (*A. castellanii*, *A. polyphaga*, and *A. quina*) and an undermined species (*Acanthamoeba* sp.)

TABLE 4. PERFORMANCE OF THE **PDH** MODEL FOR THE DIAGNOSIS OF *ACANTHAMOEBA* AND MICROSPORIDIA KERATITIS.

Positive clinical samples[a] (n=21)	Results of the PDH model		Performance of the PDH model, % (95% confidence interval)			
	Positive	Negative	Sensitivity	Specificity	PPV[b]	NPV[c]
Acanthamoeba keratitis (n=8)	7	1	87.5 (56.7–99.1)	100 (89.1–100)	100 (68.8–100)	96.2 (83.5–99.8)
Microsporidia keratitis (n=13)	13	0	100 (80.1–100)	100 (86.7–100)	100 (80.7–100)	100 (86.7–100)

[a]Positive sample as determined by the standard microbiological methods or PCR; [b]Positive predictive value; [c]Negative predictive value.

were found in the eight AK samples. This indicates a variety of *Acanthamoeba* species can infect the human eye [26].

Most AK cases are caused by wearing contact lens [6,9]. However, exposure to topical corticosteroids, contaminated soil, and water are predisposing factors of AK [27,28] and MK [7,8]. Immunocompetent and immunocompromised patients with AK and MK are at risk of developing secondary infections with other microorganisms and can progress to severe recalcitrant stromal keratitis if patients are not diagnosed early and properly treated [1,3]. As the sensitivity and specificity of the PDH model are high for diagnosis of AK and MK (Table 4), currently a multicenter study is being conducted to assess the clinical impact of this molecular technique. However, this study was unable to correlate diagnostic results with the clinical outcomes of patients because all clinical samples had been delinked with their identifiable clinical information, except their microbiological diagnostic data. In addition, this limitation also prohibits us from determining the definite final diagnoses for these eyes with negative microbiological results.

In conclusion, the PDH model developed here is a potential diagnostic tool for AK and MK. The current model can provide an alternative molecular assay for the most common parasitic keratitis. We believe this diagnostic model will facilitate early treatment, rescue vision, and minimize ocular complications due to AK and MK. However, a prospective clinical study might be needed before the model is adopted in routine clinical practice.

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