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Validation of Real-Time PCR for Laboratory Diagnosis of *Acanthamoeba* Keratitis▽

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Confirmation of *Acanthamoeba* keratitis by laboratory diagnosis is the first step in the treatment of this vision-threatening disease. Two real-time PCR TaqMan protocols (the Rivière and Qvarnstrom assays) were developed for the detection of genus-specific *Acanthamoeba* DNA but lacked clinical validation. We have adapted these assays for the Cepheid SmartCycler II system (i) by determining their real-time PCR limits of detection and amplification efficiencies, (ii) by determining their ability to detect trophozoites and cysts, and (iii) by testing a battery of positive and negative samples. We also examined the inhibitory effects of a number of commonly used topical ophthalmic drugs on real-time PCR. The results of the real-time PCR limit of detection and amplification efficiency of the Rivière and Qvarnstrom assays were 11.3 DNA copies/10 μl and 94% and 43.8 DNA copies/10 μl and 92%, respectively. Our extraction protocol enabled us to detect 0.7 *Acanthamoeba* cysts/10 μl and 2.3 *Acanthamoeba* trophozoites/10 μl by both real-time PCR assays. The overall agreement between the assays was 97.0%. The clinical sensitivity and specificity of both real-time PCR assays based on culture were 100% (7 of 7) and 100% (37 of 37), respectively. Polyhexamethylene biguanide was the only topical drug that demonstrated PCR inhibition, with a minimal inhibitory dilution of 1/640 and an amplification efficiency of 72.7%. Four clinical samples were *Acanthamoeba* culture negative and real-time PCR positive. Our results indicate that both real-time PCR assays could be used to diagnose *Acanthamoeba* keratitis. Polyhexamethylene biguanide can inhibit PCR, and we suggest that specimen collection occur prior to topical treatment to avoid possible false-negative results.

Early definitive laboratory diagnosis of *Acanthamoeba* keratitis (AK) and the prompt initiation of appropriate therapy is essential for a favorable clinical prognosis (5). Traditionally, detection of *Acanthamoeba* trophozoites and cysts in cytological preparations or growth of *Acanthamoeba* in culture have been used to diagnose AK; however, conventional PCR has been found to be more sensitive (17, 19). Real-time PCR has now emerged as an effective tool for more rapid testing of clinical samples for the detection of infectious agents (10), including *Acanthamoeba*.

Rivière et al. published the first real-time PCR study in 2006 utilizing TaqMan technology to detect *Acanthamoeba* 18S ribosomal DNA (rDNA) (14). The primers and probe were designed against six DNA sequences of *Acanthamoeba* from a single genotype (T4) common to many keratitis isolates. Development was performed on a single *Acanthamoeba* strain, and the test was not validated with any clinical samples for AK.

Qvarnstrom et al. published a later report in which real-time PCR was used as a triplex assay to detect *Balamuthia mandrillaris*, *Naegleria fowleri*, and *Acanthamoeba* species (13). The primers and probe for the *Acanthamoeba species* were designed against 40 different *Acanthamoeba* 18S rRNA sequences and tested against seven strains from four genotypes (T1, T4, T7, and T10). Qvarnstrom also evaluated the seven strains with the Rivière assay and found that it failed to detect a number of the isolates but did detect the only strain isolated from a cornea. Qvarnstrom concluded that the Rivière assay "may still be useful for detecting keratitis strains." No clinical validation was performed using clinical ocular samples.

Unlike other anatomic sites, topical medications and dyes are often administered directly to the ocular surface to assist in diagnosis and treatment. Lissamine green, rose bengal, fluorescein dyes, the anesthetic oxybuprocain, and endogenous inhibitors in ocular fluids have been shown to cause PCR inhibition (4, 16, 18), and there may be other potential inhibitors.

We sought here to (i) validate two real-time PCR methods (the Rivière and Qvarnstrom assays) for diagnosing AK using the Cepheid SmartCycler II as a real-time PCR system to detect *Acanthamoeba* DNA from ocular clinical samples and (ii) determine whether commonly used topical ophthalmic agents would affect the ability of real-time PCR to detect *Acanthamoeba* DNA. This was accomplished by (i) establishing the proficiency of two real-time PCR methods with the Cepheid SmartCycler II system in terms of limit of detection (LOD) and amplification efficiency (AE), (ii) determining the ability to detect both *Acanthamoeba* trophozoites and cysts after DNA extraction and real-time PCR testing, (iii) identifying possible topical inhibitors of real-time PCR, (iv) comparing both real-time PCR assays with a battery of positive and negative samples, and (v) validating real-time PCR for clinical application with a subset of true-positive and true-negative samples.

MATERIALS AND METHODS

Real-time PCR: establishment of proficiency. All PCRs were performed using a Cepheid SmartCycler II real-time PCR system (Cepheid, Sunnyvale, CA). Each PCR contained 15 μl of master mix (primers and/or probe and OmniMix

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TABLE 1. PCR primer and probe sequences for the development of *Acanthamoeba* real-time PCR

Pathogen	Target	Size (bp)	Concn (μM)	Primer (sequence [5′–3′])[a] and probe
Acanthamoeba spp. (Rivière assay)	18S rDNA		0.4	Forward primer TaqAcF1 (CGA CCA GCG ATT AGG AGA CG)
		65	0.4	Reverse primer TaqAcR1 (CCG ACG CCA AGG ACG AC)
			0.3	LNA TaqAcP (56-FAM/A +CA +CCA +CCA TCG GCG C/3 BHQ_1)
Acanthamoeba spp. (Qvarnstrom assay)	18S rDNA		0.4	Forward primer AcantF900 (CCC AGA TCG TTT ACC GTG AA)
		180	0.4	Reverse primer AcantR1100 (TAA ATA TTA ATG CCC CCA ACT ATC C)
			0.3	LNA AcantP1000 (5Cy5/TG +C CA +C CGA A +TA +CA/3 BHQ_2)
VZV (internal control)	ORF 38		0.3	Forward primer (AAG TTC CCC CCG TTC GC)
		82	0.3	Reverse primer (TGG ACT TGA AGA TGA ACT TAA TGA AGC)
			0.2	Probe (56-FAM-CCG CAA CAA CTG CAG TAT ATA TCG TCT CA-TAM)
Acanthamoeba spp. (conventional)	18S rDNA	470	0.3	Forward primer JDP1 (GGC CCA GAT CGT TTA CCG TGA A)
			0.3	Reverse primer JDP2 (TCT CAC AAG CTG CTA GGG GAG TCA)
Acanthamoeba spp. (sequencing primers)	18S rDNA		0.0034	892 (CCA AGA ATT TCA CCT CTG AC)
			0.0034	892C (GTC AGA GGT GAA ATT CTT GG)

[a] +, Position of LNA.

HS beads [TaKaRa Bio, Inc., Otsu, Shiga, Japan]) and 10 μl of test or control sample placed in 25-μl SmartCycler II reaction tubes. The Qvarnstrom assay was modified from its original triplex format for optimization as a singleplex assay. The real-time PCR settings were as follows: 95°C for 2 min (Hot Start *Taq* polymerase activation), followed by 45 cycles of 95°C for 15 s (denaturing), 55°C for 30 s (Qvarnstrom annealing) or 60°C for 30 s (Rivière and varicella-zoster virus [VZV] annealing), and 72°C for 30 s (extension).

Table 1 presents the primers and probes used for the detection of *Acanthamoeba* spp. (Rivière and Qvarnstrom assays), VZV internal control (for inhibition control for clinical validation), conventional PCR primers (for amplification of *Acanthamoeba* DNA without probe for amplicon sequencing), and *Acanthamoeba* sequencing primers (2). Locked nucleic acids (LNA) were used for the *Acanthamoeba* real-time PCR probe construction for increased binding affinity (11).

The AEs and the LODs of the Rivière and Qvarnstrom real-time PCR assays were determined by using serial dilutions of respective cloned plasmid DNA. The plasmid DNA was prepared by using the pGEM-T Easy Vector system (Promega Corp., Madison, WI). The plasmid DNA was purified by using a Wizard Miniprep kit (Promega Corp., Madison, WI) and quantified by using a spectrophotometer (Beckman Coulter, Inc., Fullerton, CA). Linear regression was used to determine the slope (Minitab, Inc., State College, PA) of the cycle threshold (C_T) versus the target DNA dilution. The AE was calculated by using the equation: $E = 10^{-1/\text{slope}} - 1$. An AE between 90 and 105% was considered efficient.

The DNA extraction method was used in three capacities: (i) to evaluate the ability to extract DNA from *Acanthamoeba* trophozoites and cysts, (ii) to extract plasmid DNA from any drug-inhibited samples, and (iii) to extract *Acanthamoeba* DNA from clinical samples. The DNA extraction was performed by using the MasterPure complete DNA and RNA purification kit (Epicentre, Madison, WI) using the manufacturer's recommendations (9). In brief, specimens for PCR processing were prepared by heating 0.3 ml of sample for 10 min at 98°C and placing them on ice. (Heating helps to lyse cells to expose DNA). The protein was extracted from boiled samples with 0.15 ml of MasterPure complete protein precipitation solution. The mixture was vortex mixed for 10 s and centrifuged at 10,000 rpm for 10 min at 4°C. (The extraction step is necessary to eliminate any inhibitory effect from the presence of protein and to remove any fluorescein that is used during the clinical examination. The high background provided by the residual fluorescein interferes with the fluorescent signal of the PCR product.) The supernatant was transferred to a clean tube. The DNA was precipitated from the supernatant by adding 0.5 ml of isopropanol (DNase, RNase, Protease-free; Acros Organics/Fisher Scientific, Pittsburgh, PA), inverting the tube 30 to 40 times, and centrifuging the contents at 10,000 rpm for 10 min at 4°C. The supernatant was discarded, and the pellet containing the DNA was washed twice with 75% ethanol (0.5 ml) using centrifugation at 10,000 rpm for 5 min at 4°C. The remaining pellets were dried under vacuum to eliminate any residual ethanol that might inhibit the PCR. The final DNA pellet was suspended in 35 μl of TE buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA; Epicentre) (10).

Detection of *Acanthamoeba* trophozoites and cysts. The ability to detect both *Acanthamoeba* trophozoites and cysts was determined by DNA extraction and real-time PCR testing.

Acanthamoeba polyphaga (ATCC 30461) trophozoites were grown in yeast extract-peptone-dextrose (YPD) broth (Teknova, Inc., Hollister, CA) until the flask was confluent (24 to 48 h) (8). The YPD broth was removed and the trophozoite monolayer was washed twice with fresh YPD to remove any cysts. The flask was placed in ice for 1 h to detach the trophozoites (modified from the method of Qvarnstrom et al. [13]). The contents of the flask were transferred to a chilled 15-ml Falcon tube and centrifuged for 5 min at 2,500 rpm at 4°C. The supernatant was removed, and trophozoites were washed in 2.0 ml of chilled saline (0.85% NaCl) and centrifuged for 5 min at 2,500 rpm at 4°C. The trophozoites were washed again in saline, centrifuged, and decanted, and 2.0 ml of chilled Bartels chlamydial transport media (CTM; Bartels, Bellevue, WA) was added. The trophozoites were adjusted to 10^4 trophozoites/ml by using a hemacytometer (Bright-Line, Buffalo, NY) and serially diluted twofold to less than one trophozoite per 300-μl sample in CTM. The DNA extraction was performed as previously described. Portions (10 μl) of extract were used in the real-time PCR procedure.

For cysts, *A. polyphaga* (ATCC 30461) trophozoites were grown in YPD broth until the flask was confluent (24 to 48 h). To induce encystment, the YPD was decanted, and 10 ml of RPMI 1640 with 8% glucose (Invitrogen Corp., NY) was added. The flask was incubated at 30°C for 48 h. To ensure that only cysts were present, sodium dodecyl sulfate (0.5% final concentration) was added (sodium dodecyl sulfate lyses the trophozoites) (8). The contents of the flask were transferred to a 15-ml Falcon tube and centrifuged at 2,500 rpm for 5 min. The medium was decanted, and the cysts were washed in 2.0 ml of saline, followed by centrifugation at 2,500 rpm for 5 min. The saline was decanted, and the cysts were washed again in 2.0 ml of 0.85% saline and centrifuged. The step was repeated, and 2 ml of CTM was added. The cysts were adjusted to 10^4 cysts/ml by using a hemacytometer and serially diluted twofold down to less than one cyst per 300-μl sample in CTM. The DNA extraction was performed as previously described. Portions (10 μl) of the extract were used in the real-time PCR procedure.

Determination of the minimal inhibitory drug dilution using real-time PCR. To determine the minimal inhibitory drug dilution of topical ophthalmic drugs on real-time PCR, 1 μl of Rivière *Acanthamoeba* plasmid DNA (1.13×10^5 copies/μl) was pipetted to the serial dilutions of 0.5% proparacaine hydrochloride (Proparacaine; Falcon Pharmaceuticals, Fort Worth, TX), 0.3% gatifloxacin (Zymar; Allergan, Irvine, CA), 0.5% moxifloxacin hydrochloride (Vigamox; Alcon Laboratories, Fort Worth, TX), 1% prednisolone acetate (Econopred Plus; Alcon Laboratories), 0.09% bromfenac (Xibrom; ISTA Pharmaceuticals, Irvine, CA), 0.1% nepafenac (Nevanac, Alcon Laboratories), 1.4% tobramycin (MP Biomedicals, Solon, OH), 5.0% cefazolin (MP Biomedicals), 0.15% amphotericin B (MP Biomedicals), 1% trifluridine (Trifluridine; Falcon Pharmaceuticals), 0.1% propamidine isethionate (Brolene; Aventis Pharma, Auckland, New Zealand), 0.02% polyhexamethylene biguanide (PHMB; Leiter's Pharmacy, San Jose, CA), 0.02% chlorhexidine (Leiter's Pharmacy), and corneal rim tissue. The corneal rims represent excess tissue obtained from keratoplasty cases after routine bacterial culture. They were de-identified and homogenized prior to assay. Each of the ophthalmic drugs was serially diluted with TE buffer to include dilution factors of 1 to 320 (640 to 40,960 as needed).

Drugs that inhibited the real-time PCR at high levels were retested following a DNA extraction to ascertain if the inhibitory effects could be eliminated.

Effect of minimal inhibitory drug dilution on real-time PCR AE. To determine the effect of the minimal inhibitory drug dilution on real-time PCR AE, 10-fold serial dilutions (10^{-3} to 10^{-9}) of concentrated Rivière *Acanthamoeba* plasmid DNA (381.2 μg/ml) were prepared using each specific drug (at its minimal inhibitory titer) as the diluents. The regression plot and AE were determined from the real-time PCR as previously described.

TABLE 2. Descriptive statistics of *Acanthamoeba* DNA detection by real-time PCR

Parameter	Assay	
	Rivière	Qvarnstrom
Lowest dilution (plasmid DNA)	10^{-11}	10^{-9}
LOD (mean no. of copies/10 μl)	11.3	43.8
LOD (mass [ag])	38.12	157.75
Correlation coefficient r^2 (%)	99.8	99.2
AE (%)	94	92
LOD[a] (mean no. of trophozoites/10 μl)	2.3	2.3
LOD[a] (mean no. of cysts/10 μl)	0.7	0.7

[a] Data represent the results of two experiments.

Comparison of the Rivière and Qvarnstrom real-time PCR methods. The Rivière and Qvarnstrom real-time PCR methods were compared by using a battery of positive, negative, and unknown samples.

The positive samples were comprised of seven in-house *Acanthamoeba* strains collected from AK. These isolates were de-identified, collected as excess specimens, and stored at 4°C for validation of clinical testing. Another seven strains were purchased through the University of Pittsburgh from the American Type Culture Collection (ATCC): *Acanthamoeba lugdenensis* ATCC 24050 (T4), *A. polyphaga* ATCC 30461 (T4), *A. hatchetti* ATCC PRA-113 (T11), *A. castellanii* ATCC 30010 (T4), *A. rhysodes* ATCC 50368 (T4), *A. culbertsoni* A-1 ATCC 30171 (T10), and *A. griffini* TIO:H37 ATCC 50702 (T3). All isolates were propagated axenically in 25-cm^2 tissue culture flasks containing YPD broth. The strains were cultivated at 30°C in an aerobic incubator to attain 40 to 50% growth, and then 300 μl of the YPD broth was removed for DNA extraction.

The negative samples consisted of 37 non-*Acanthamoeba* pathogens that can be associated with keratitis. The isolates were de-identified, collected as excess specimens, and stored at −80°C for validation of clinical testing. The bacterial, fungal, and viral isolates (*Staphylococcus aureus* [$n = 4$], *Pseudomonas aeruginosa* [$n = 4$], *Haemophilus influenzae* [$n = 3$], *Streptococcus pneumoniae* [$n = 3$], nutritionally variant streptococcus [$n = 2$], *Streptococcus viridans* [$n = 2$], *Moraxella* species [$n = 1$], *Mycobacterium chelonae* [$n = 1$], *Serratia marcescens* [$n = 1$], *Escherichia coli* [$n = 1$], *Bacillus* species [$n = 1$], *Nocardia farcinica* [$n = 1$], *Fusarium* species [$n = 1$], *Alternaria* species [$n = 1$], *Penicillium* species [$n = 1$], *Aspergillus niger* [$n = 1$], adenovirus [$n = 3$], herpes simplex virus type 1 [$n = 4$], and VZV [$n = 1$]) were prepared as previously published (10). A single isolate of *Hartmannella* species was grown axenically as described for *Acanthamoeba* propagation.

A collection of specimens from patient sources with a differential of AK were also tested by both methods. These retrospective clinical samples for the real-time PCR validation testing were de-identified, collected as excess specimens, and stored at −80°C. These samples were collected for viral testing and/or *Acanthamoeba* culture. No additional samples were collected for the sole purpose of real-time PCR testing. Ocular samples were collected with a sterile soft-tipped applicator or by scraping the area of infection with a spatula and placing the collection in 2.0 ml of CTM. Tissue or contact lens (CL) paraphernalia for *Acanthamoeba* culture were inoculated directly onto non-nutrient agar plates overlain with a fresh suspension of *Enterobacter aerogenes* (ATCC 35028) in saline (0.85% NaCl) and incubated at 30°C for up to 7 days. CL cases for *Acanthamoeba* real-time PCR were collected by saturating a sterile soft-tipped applicator in the case solution and placing the applicator into 2.0 ml of CTM. CL solution bottles were aliquoted into 15-ml Falcon tubes and centrifuged at 3,500 rpm for 10 min. The supernatant was decanted, and a sterile soft-tipped applicator was used to absorb any liquid precipitate. The swab was placed into 2.0 ml of CTM, and DNA was extracted. All samples collected from patient sources were tested concurrently for PCR inhibitors using a VZV internal control (Table 1).

Acanthamoeba culture-negative samples that were *Acanthamoeba* real-time PCR positive were amplified with conventional PCR (Table 1) and sequenced, and the results compared to catalogued *Acanthamoeba* sequences in GenBank (National Center for Biotechnology Information, Bethesda, MD).

Validation of the real-time PCR assays for clinical testing. The validation of real-time PCR for both assays was determined by using the seven *Acanthamoeba* culture-positive specimens (true positives) collected directly in CTM from patients and the 37 non-*Acanthamoeba* isolates (true negatives) in CTM. Patient samples collected with a differential diagnosis of AK, although *Acanthamoeba* culture negative, were not considered true negatives because of the possible presence of DNA.

TABLE 3. Summary of minimal inhibitory drug dilutions and AEs of topical ocular drugs

Drug	Minimal inhibitory dilution	AE
Proparacaine (anesthetic)	1/1	100.1
Prednisolone (steroid)	1/1	91.2
Bromfenac (nonsteroidal)	1/1	97.4
Nepafenac (nonsteroidal)	1/10	95.0
Gatifloxacin (antibiotic)	1/10	92.6
Moxifloxacin (antibiotic)	1/10	92.0
Tobramycin (antibiotic)	1/1	102.9
Cefazolin (antibiotic)	1/10	91.6
Amphotericin B (antifungal)	1/1	108.7
Trifluridine (antiviral)	1/40[a]	94.6
Propamidine (antiacanthamoebic)	1/10	85.7
Polyhexamethylene biguanide (antiacanthamoebic)	1/640[b]	72.7
Chlorhexidine (antiacanthamoebic)	1/10	77.0
Corneal tissue	1/1	86.9

[a] Trifluridine inhibition was removed completely after DNA extraction (1/1).
[b] Polyhexamethylene biguanide inhibition was still present after DNA extraction (the minimal inhibitory dilution improved onefold to 1/320).

RESULTS

Proficiency of the Rivière and Qvarnstrom real-time PCR assays. The LOD of the Rivière assay was 11.3 DNA copies/10 μl or 38.12 ag of DNA. The AE was 94% determined from a regression with a correlation coefficient (r^2) of 99.8% and a slope of −3.47196 (Table 2).

The LOD of the Qvarnstrom assay was 43.8 DNA copies/10 μl or 153.75 ag of DNA. The AE was 92% determined from a regression with a correlation coefficient (r^2) of 99.2% and a slope of −3.53123 (Table 2).

Detection of *A. polyphaga* (ATCC 30461) trophozoites and cysts. After the DNA extraction and real-time PCR, the LODs for both assays using trophozoites were determined to be the same at 2.3 ± 1.7/10 μl (Table 2). The LODs for both assays using cysts were determined to be the same at 0.7 ± 0.0 cysts/10 μl (Table 2).

Determination of the minimal inhibitory drug dilution by using real-time PCR. The minimal inhibitory dilutions of 12 commonly used topical ophthalmic drugs are listed in Table 3. Two drugs showed elevated minimal inhibitory drug dilutions. Trifluridine demonstrated a minimal inhibitory drug dilution of 1/40; however, all inhibitory effects were eliminated after DNA extraction. PHMB demonstrated an even greater inhibitory effect (1/640) but only improved by onefold after the DNA extraction to 1/320.

Based on an AE greater than 90%, the minimal inhibitory drug concentrations of proparacaine hydrochloride, prednisolone acetate, bromfenac, tobramycin, nepafenac, gatifloxacin, moxifloxacin hydrochloride, cefazolin, and trifluridine did not adversely affect PCR (Table 2). The corneal tissue, amphotericin B, propamidine isethionate, and chlorhexidine were determined to have AEs just outside the recommended range and thus could have some adverse effects on the PCR. These effects may be eliminated at higher dilutions. The AE of PHMB was 72.7%; thus, PHMB was deemed adverse to PCR amplification.

TABLE 4. Comparison of two real-time PCR assays for the detection of *Acanthamoeba* DNA[a]

Rivière real-time PCR result	Qvarnstrom real-time PCR result (no. of isolates)				
	Positive	Negative	Indeterminate	Inhibitory	Total
Positive	11[b]	2[c]	0	0	13
Negative	0	149[d]	0	0	149
Indeterminate	0	3[e]	1[f]	0	4
Inhibitory	0	0	0	2[g]	2
Total	11	154	1	2	168

[a] "Indeterminate" indicates a sample that tested positive initially, but the result was not reproducible. "Inhibitory" indicates samples where the internal control tested negative after extraction and re-extraction.
[b] Nine cornea and two CL cases. Seven were culture positive, real-time PCR positive, and four were culture negative, real-time PCR positive.
[c] One cornea, one CL solution bottle.
[d] 95 cornea, 32 CL cases, 20 CL solution bottles, 1 eyelid, 1 conjunctiva and eyelid.
[e] Two cornea, one CL case.
[f] One CL case.
[g] Two CL cases.

TABLE 5. Clinical validation statistics for *Acanthamoeba* DNA detection

Parameter[a]	Assay	
	Rivière	Qvarnstrom
True-positive isolates	7	7
True-negative isolates	37	37
Sensitivity (%)	100.0	100.0
Specificity (%)	100.0	100.0
PPV (%)	100.0	100.0
NPV (%)	100.0	100.0
Efficiency (%)	100.0	100.0

[a] True positives were direct patient specimens that were *Acanthamoeba* positive in culture. True negatives were samples of non-*Acanthamoebic* pathogens in chlamydial transport medium. PPV, positive predictive value; NPV, negative predictive value.

Comparison of the Rivière and Qvarnstrom real-time PCR methods. (i) Negative controls and positive controls. The seven in-house keratitis isolates and the seven ATCC strains of *Acanthamoeba* were positive by the Qvarnstrom assay; however, only 13 of 14 were determined to be positive by the Rivière assay. The negative result was *A. culbertsoni* A-1 ATCC 30171. All 37 non-*Acanthamoebic* pathogens were determined to be negative by both assays.

(ii) Samples from patient sources. A total of 168 ocular clinical samples were tested for *Acanthamoeba* DNA. The sources of the samples were as follows: 107 (63.7%) were cornea samples, 38 (22.6%) were CL cases, 21 (12.5%) were from CL solution bottles, 1 (0.6%) was derived from an eyelid sample, and 1 (0.6%) was derived from both an eyelid and conjunctiva sample. The two assays demonstrated 97% (163 of 168) agreement. Of the five contrasting samples, two were determined to be positive by the Rivière real-time PCR but negative by the Qvarnstrom real-time PCR, and three were indeterminate by the Rivière real-time PCR but negative by the Qvarnstrom real-time PCR. Table 4 summarizes the results of both assays.

The four culture-negative and PCR-positive samples were sequenced and confirmed to be *Acanthamoeba* DNA using the GenBank database (data not shown).

Validation of the real-time PCR assays for clinical testing. Table 5 summarizes the descriptive statistics of clinical validation of real-time PCR by both assays. Seven clinical samples (six cornea samples and one CL case) were positive for *Acanthamoeba* culture and real-time PCR by both assays. Thirty-seven non-*Acanthamoebic* pathogens were all determined to be negative by both real-time PCR assays.

DISCUSSION

The number of potentially vision-threatening cases of AK may be on the rise, and this increase may, in part, be due to contamination of a commonly used CL solution (1, 6, 7). The number of requests for *Acanthamoeba* culture has increased fivefold over the past 4 years, i.e., 2004 to 2008, at the Ophthalmic Microbiology Laboratory of the University of Pittsburgh Medical Center (unpublished data). Real-time PCR for the detection of *Acanthamoeba* DNA from ocular specimens can supplement the traditional culture technique, but testing needs to be highly sensitive and tolerant of amplification inhibitors and be able to detect all strains of *Acanthamoeba*. The present study compared the most recognized real-time PCR TaqMan assays (Rivière and Qvarnstrom) to determine whether one assay was better in detecting *Acanthamoeba* DNA from clinical ocular samples using the Cepheid SmartCycler II system. Both assays performed comparably in terms of their ability to detect low numbers of *Acanthamoeba* DNA copies in light of *Acanthamoeba* containing more than 600 copies within its ribosomal gene repeat unit (3). Both assays demonstrated no cross-reaction with other ocular pathogens. The Qvarnstrom assay was able to detect 14 of 14 *Acanthamoeba* strains, while the Rivière assay only detected 13 of 14. The undetected strain was a T10 strain (*A. culbertsoni* A-1 ATCC 30171) which has not been associated with keratitis. On investigation, Rivière's reverse primer sequence (TaqAcR1) shares little homology with the GenBank sequence *A. culbertsoni* AF019067 (not shown).

For purposes of comparison, 168 ocular clinical samples were tested, of which 163 results demonstrated overall agreement (97.0%) between the two assays. Four samples were culture negative, real-time PCR positive and were subsequently confirmed by DNA sequencing. Of the five contrasting samples, the discrepancy may simply be due to low DNA copy number and random sampling error.

In comparing the development of both assays, the Qvarnstrom assay is the more complete of the two because it was designed to detect a broader range of *Acanthamoeba* genotypes. A limitation of the Rivière assay is that its design was based on the T4 genotype (the most common *Acanthamoeba* isolate in AK). However, the literature reveals that the T2a, T3, T6, and T11 genotypes have also been associated with AK (8). AK from these and other genotypes is possible because of *Acanthamoeba*'s ubiquity in the environment (12) and the increased risks associated with the increased use of CLs (15).

An important aspect of any PCR testing is the use of an appropriate DNA extraction procedure. In testing *Acanthamoeba*, there is the added challenge of breaking open a trophozoite or cyst to efficiently access its genomic material.

Using a commercial extraction technique, we were able to detect low numbers of both trophozoites and cysts.

Topical ophthalmic medications may be inhibitory to PCR amplification and lead to false-negative results that can affect proper diagnosis and treatment (4, 16). In testing 12 topical ophthalmic drugs for inhibitory factors, only PHMB, a commonly used biocide used to treat AK, inhibited the real-time PCR at high dilutions despite having an extraction procedure. This supports the point that diagnostic specimens should be collected before treatment with PHMB is initiated to optimize the detection of *Acanthamoeba* DNA by PCR testing.

In conclusion, we have established and validated two real-time PCR assays for the detection of *Acanthamoeba* DNA from clinical ocular samples using the Cepheid SmartCycler II real-time PCR system. The administration of most commonly used topical ocular drugs does not appear to be a major factor of PCR inhibition, except for PHMB, which should be withheld until after specimen collection has occurred. Clinical judgment, along with routine cultures, smears, and PCR testing for *Acanthamoeba* detection, should be tools to guide the ophthalmologist in the treatment of this potentially blinding disease.

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