A One-Step Multiplex PCR for *Acanthamoeba* Keratitis Diagnosis and Quality Samples Control

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Purpose. As the number of cases of *Acanthamoeba* spp. keratitis (AK) is constantly growing, new diagnostic tools are needed to confirm and guide ophthalmologists in this clinically problematic diagnosis. Molecular diagnosis is particularly well adapted, although only a few real-time PCR techniques have been described recently. The aim of this study was to develop a new PCR technique for the diagnosis of AK by combining the detection of *Acanthamoeba* DNA with human DNA, thus allowing an accurate interpretation of the PCR result.

Methods. Different DNA extraction procedures were compared to ensure an optimized amplification of one *Acanthamoeba* genome. The analytical parameters of this new multiplex *Acanthamoeba* beta-globin PCR (MAB-PCR) were evaluated. Fourteen eye drops were tested as potential PCR inhibitors. A prospective series of 28 corneal scrapings was subjected to MAB-PCR.

Results. The best extraction procedure associated thermal-shock pretreatment followed by a manual extraction procedure. The MAB-PCR parameters displayed excellent specificity and sensitivity, with a detection of 0.02 genome of *Acanthamoeba*. No eye drops were total PCR inhibitors. Of 28 corneal scrapings, 18 were considered true negatives. Seven could not be interpreted because of insufficient scraping material. Three were considered true positives: every patient progressed favorably on specific and reliable treatment.

Conclusions. The MAB-PCR is a new tool to diagnose AK. It allows rapid diagnosis and prompt treatment of this probably underestimated etiology of infectious keratitis. This optimized real-time PCR outperforms the gold standard for *Acanthamoeba* keratitis diagnosis and it allows a concomitant evaluation of the quality of the corneal scraping, which is necessary for a precise interpretation of the results. (*Invest Ophthalmol Vis Sci.* 2012;53:2866–2872) DOI:10.1167/iovs.11-8587

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Among the etiological agents responsible for infectious keratitis, the free-living amoeba of the *Acanthamoeba* spp. appears to be an emerging pathogen because of its frequent association with contact lens wear.[1–4] *Acanthamoeba* keratitis (AK) is a serious sight-threatening disease.[2] Differential diagnoses are herpes keratitis in its early aspect or fungal keratitis in its later aspect. Because clinical diagnosis of *Acanthamoeba* can be challenging, and prognosis is directly related to timely diagnosis,[5,6] a method to ensure rapid and specific diagnosis is needed.

The most commonly used diagnostic procedure is based on the association of a microscopic examination of the corneal scraping and a culture procedure. This strategy is far from being adapted to the urgency of the situation because the complete procedure requires 21 days of culture and presents a very low overall sensitivity (50% misdiagnosed cases).[7] This lack of sensitivity may stem from the culture procedure, but also the difficulty of obtaining sufficient corneal scraping material. Over the past 10 years, new diagnostic strategies have been developed for AK. Molecular biology and more precisely PCR procedures amplifying *Acanthamoeba* DNA have been developed to improve AK management, but the number of these PCR procedures remains quite low.[8–12] PCR procedures have proved their efficiency in diagnosing AK, with a real benefit for patient care; however, the sensitivity in diagnosing AK with a molecular diagnostic procedure can still be unsatisfactory because of insufficient DNA material on the corneal scraping and the resistance of the cystic form to conventional DNA extraction methods.[13]

With this in mind, we multiplexed the amplification of the rRNA 18S gene of *Acanthamoeba* spp. with the human beta-globin gene. The objective of this new tool was to inform clinicians on the presence of *Acanthamoeba* DNA, but also on the quality of the corneal scraping and any presence of PCR inhibitors. Different pretreatment and extraction procedures were also compared to optimize the extraction of *Acanthamoeba* cysts. Multiplex *Acanthamoeba* beta-globin PCR (MAB-PCR) was tested prospectively on a series of patients with suspected infectious keratitis. This study aimed to confirm that PCR diagnosis is highly advantageous for AK diagnosis, but it also intended to contribute supplemental information on the quality of the scraping to ensure optimal use and interpretation of the PCR diagnosis in this particular context.

Materials and Methods

Acanthamoeba Strains and Human Cells

Table 1 describes the different *Acanthamoeba* strains tested in this study. *Acanthamoeba polyphaga* reference strain[14] was used for the DNA extraction optimization step and the validation of the PCR assays. Clinical in-house isolates 1, 2, 3, and 4 from different genotypes, were

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Table 1. Genotype Study of the Different *Acanthamoeba* spp. Isolates of the Study

Name	Genotypes
Reference strain *A. polyphaga*	T4
Culture of in-house isolate 1	T3
Culture of in-house isolate 2	T6
Culture of in-house isolate 3	T4
Culture of in-house isolate 4	T4

tested subsequently in the same manner. All strains were maintained in their vegetative form in a culture flask at 27°C in peptone–yeast extract–glucose medium, as previously described.[15] The vegetative form was converted to the cystic form as described before.[10] All strains were genotyped analyzing the ASA.S1 region of the *Acanthamoeba* rRNA 18S gene.[16] Cysts or vegetative forms were numbered in a cell-counting chamber, but for isolation of only one cyst, 50 μL of a solution of 20 cysts/mL was dispensed in a 96-well plate and screened under an inverted microscope for wells containing one cyst.

Human cells were obtained from human foreskin fibroblast (HFF) monolayer culture. Individualized HFF cells were suspended after trypsinization of the monolayer and the cells were counted with a cell-counting chamber.

Sample Pretreatment and DNA Extraction Procedure

Samples must be pretreated to ensure lysis of the cyst cell wall. Procedures are mostly based either on chemical lysis[17] or mechanical lysis based on the thermal-shock procedure.[9,13,18] These two procedures were compared. *Amoeba* cysts or trophozoites were suspended in 100 μL of PBS, 100 μL of the detergent solution (Tris-HCl 20 mM and SDS 0.5%), and 10 μL of proteinase K (20 minutes at 56°C, then 10 minutes at 95°C), or in 180 μL of a tissue lysis buffer solution (Qiagen, Hilden, Germany) added to 20 μL of proteinase K, incubated for 10 minutes at 56°C, then thermally shocked (10 minutes at 95°C, 3 minutes at −80°C, 1 minute 95°C) and extracted.

Two different extraction procedures were also tested: one based on a manual procedure (Qiagen QIAamp DNA Mini kit) and another using automatic extraction (BioRobot EZ1 Workstation, with EZ1 DNA Tissue card, Qiagen). DNA was eluted in 50 μL of elution buffer AE (Qiagen). PCR was performed on 12 samples containing one cyst submitted to each different pretreatment; on 20 samples containing one, two, or three cysts; and 12 samples containing one, two, or three vegetative forms of *A. polyphaga*, all thermally shocked but extracted with two different procedures.

Primers and Probes, PCR Procedure

Table 2 describes the sequence of all primers used in the multiplex PCR assays. Primers and the hydrolysis probe (AcantF, AcantR, AcantP) were those described previously: these primers were proved to amplify the majority of *Acanthamoeba* spp. genotypes involved in AK.[9,12] A systematic search with the Basic Local Alignment Search Tool (BLAST) was done on the 17 different genotype sequences and showed that primers and probe present 100% homology with 14 of the 17 genotypes, including all the genotypes commonly involved in eye pathologies (i.e., T4, T3, T6, and T11) (see Supplementary Material, available at http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-8587/-/DCSupplemental).

The primers and a hydrolysis probe to amplify human DNA were designed using Primer3 software[19] to amplify a region of the human beta-globin gene.

The PCR mix was composed of SmartMix HM reagent (Cepheid, Sunnyvale, CA), 10 mM of Tris-HCl, 0.3 μM of AcantP, 0.2 μM of βGlob probe, and 0.4 μM of each primer and 10 μL of the DNA sample in each PCR tube. The final volume was 25 μL for one reaction. The PCR amplification procedure was carried out on a SmartCycler Robot (Cepheid) and the cycling protocol was adapted to fit with both *Acanthamoeba* and beta-globin sequence amplification: 2 minutes at 95°C (hot-start, one cycle), followed by 45 cycles at 95°C for 15 seconds, 60°C for 30 seconds, and 72°C for 30 seconds.

Positive control DNA for *Acanthamoeba* was obtained from 2.10^6 cysts of *A. polyphaga*–encysted culture by using the optimal pretreatment and extraction procedure and serially diluted. The positive control for beta-globin was obtained from a suspension of 10^5 HFF cells using the same procedure.

Validity of the PCR Protocol

The validity of the MAB-PCR was assessed according to the minimum information for publication of quantitative real-time PCR experiments.[20]

The analytical sensitivity and the limit of detection (LOD) were first determined by the number of the *Acanthamoeba* genomes detected with the overall experimental assay (including sample pretreatment and DNA extraction). As the corneal scraping can be very poor in pathogenic material, we aimed to ensure effective amplification of at least one genome of *Acanthamoeba*. We therefore isolated one cyst of *A. polyphaga* (see previously described procedure), which underwent the entire PCR assay (optimal pretreatment and extraction procedure followed by PCR amplification). A total of 31 isolated cysts were extracted and amplified in eight different experiments.

The linear dynamic range was evaluated over a 5 $\log_{10}$ concentration on a serial dilution from 1000 to 0.1 genomes of *A. polyphaga*, corresponding to 200 to 0.02 genome/PCR reaction tube.

To assess the analytical specificity in vitro, ensuring that the MAB-PCR procedure detects no unspecific targets, PCR was evaluated on different microbial pathogens that are commonly isolated in the context of infectious keratitis, that is, for bacteria: *Staphylococcus aureus, Staphylococcus epidermidis, Pseudomonas aeruginosa, Streptococcus pneumoniae, Haemophilus influenzae, Serratia marcescens, Corynebacterium* sp., *and Propionibacterium acnes*; for fungi: *Candida albicans, Candida parapsilosis, Aspergillus fumigatus*, and *Fusarium* sp. Monomorphic culture of each of these microorganisms was extracted with the manual DNA extraction kit (Qiagen QIAamp DNA Mini kit). To ensure that a sufficient amount of DNA was extracted, DNA was quantified by spectrophotometry (NanoDrop; 2000C Thermo Scientific, Waltham, MA) and MAB-PCR was completed.

The intra-assay and inter-assay variances were completed on four different DNA concentrations obtained after extraction of 1, 10, 100, and 1000 cysts associated with 50 human cells.

Table 2. Primers and Probes of the MAB-PCR

Genes	Names	Sequences 5′–3′
Acanthamoeba spp. rDNA (18S)	Primer Acant-F	CCCAGATCGTTTACCGTGAA
	Primer Acant-R	TAAATATTAATGCCCCCAACTATCC
	Probe Acant-P	5′[Cy5]TG[+C]CA[+C]CGAA[+T]A[BHQ2]
Human beta-globin	Primer βGlob-F	TGAGTCTATGGGACGCTTGA
	Primer βGlob-R	AAAAATTGCGGAGAAGAAAAA
	Probe βGlob-P	[FAM]TCCTGAGACTTCCACACTGATGC[BHQ1]

Search for Potential PCR Inhibitors

Previous studies have shown that some eye drop solutions can interfere with the PCR procedure.[9,21] Fourteen of the eye drops most commonly used were tested as potential inhibitors of the MAB-PCR. Considering that most of the DNA extraction kits are assumed to remove most inhibitors, we chose to evaluate this inhibitor effect on samples that had gone through the whole extraction procedure (as would a real corneal scraping sample). Because the average volume of an eye drop is 40 μL, we estimated that no more than the equivalent of 8 μL of eye drop solution could be found in a corneal scraping. Thus, 8 μL of each tested solution was added to a PBS solution containing one isolated cyst of *A. polyphaga* and 100 human cells, and the extracted DNA sample was tested for MAB-PCR. The cycle quantification (Cq) for each sample was compared with the mean Cq of the control (either one cyst or 100 human cells). A solution was considered a total PCR inhibitor when no amplification was obtained or a partial inhibitor when the Cq was higher than the control Cq + 2 SD. All samples were tested in duplicate.

Corneal Scraping Samples

To test the diagnostic sensitivity of the MAB-PCR, corneal scraping samples usually dedicated for conventional AK diagnostic were used. Samples were obtained from 28 patients with suspected infectious keratitis requiring deep scraping of the cornea under slit lamp, with sterile stainless steel blades, before institution of therapy. The study was conducted in accordance with the Declaration of Helsinki for research involving human subjects and adhered to Good Practice guidelines. Informed consent for the corneal scraping was obtained from the subjects after explanation of the technique and the potential benefit of microbiological diagnosis.

When two scrapings were received, one was dedicated to the PCR and the other to conventional culture (non-nutrient agar plates overlain with a fresh suspension of *Escherichia coli* (ATCC 25922) and incubated at 27°C for up to 21 days). The pretreatment chosen was applied directly to the scalpel within the collection tube. When only one scraping was received, samples were suspended in two drops of DNA-free distilled water and split in two so that the MAB-PCR could be done. As the yield of extracted DNA was theoretically low, a DNA carrier (Poly(A); Roche Applied Science, Penzberg, Germany) was systematically added to the sample before using the optimal extraction procedure. Each corneal scraping extracted DNA was tested in triplicate: a duplicate containing the sample DNA and a third tube containing sample DNA added with 1 μL of the positive *Acanthamoeba* sample as internal control for inhibitor detection.

Statistical analysis was done using Statview 5.0 software (SAS Institute, Cary, NC). Because of the small sampling size, we used nonparametric tests, the Fisher exact test, or the Mann-Whitney *U* test. *P* values under 0.05 were considered statistically significant.

Results

Sample Pretreatment and DNA Extraction Procedure

A positive PCR result was obtained in 3 (25%; mean Cq, 37.89 ± 5.34) of 12 samples prepared with the chemical lysis solution and in 6 (50%; mean Cq, 35.09 ± 2.06) of 12 samples prepared with the thermal-shock procedure. These results prompted us to choose the thermal-shock procedure as pretreatment ($P = 0.4$, Fisher exact test).

The mean Cq obtained after the thermal-shock procedure, with the manual extraction procedure and the automatic method were compared. Figure 1 shows that the mean Cq with the manual method was significantly lower than the mean Cq obtained after automatic extraction ($P = 0.006$ and 0.0003, respectively, for cysts and vegetative forms, using the Mann-Whitney test). In the end, the best extraction procedure for *Acanthamoeba* spp. was the thermal-shock pretreatment followed by the manual DNA extraction procedure (Qiagen); this specific protocol was used to conduct all subsequent experiments.

Validity of the PCR Protocol

Analytical Sensitivity and LOD: Of the 31 extracted samples containing one cyst, 30 samples showed a positive result with

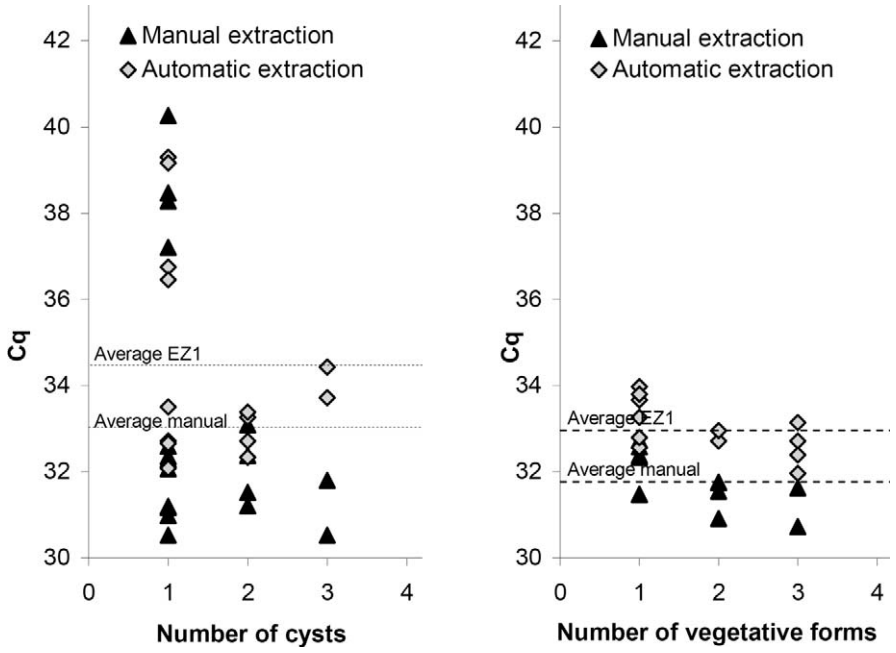


Figure 1. Cq values with different concentrations of amoeba and different extraction methods. For cysts: mean Cq for the manual method: 33.02 (±3.06) and for EZ1 automatic method: 34.46 (±2.14). For vegetative forms: mean Cq for manual method: 31.77 (±0.63) and mean Cq for EZ1 method: 32.99 (±0.6).

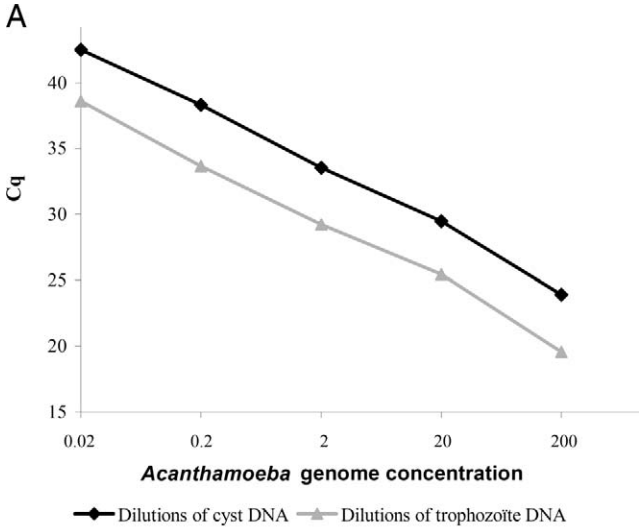
the MAB-PCR assay (mean Cq, 33.48 ± 2.36). The analytical sensitivity of the entire PCR assay (including pretreatment and extraction) was evaluated at 97% for one cyst. The acceptable LOD of the entire assay was at least one cyst.

The *linear dynamic range* was evaluated on a serial dilution of *Acanthamoeba* DNA, extracted from a suspension containing 1000 cysts or vegetative forms of *A. polyphaga*. Figure 2A shows the linear dynamic range over a 5 $\log_{10}$ concentration of the *A. polyphaga* genome (from 200 to 0.02 genome). The correlation coefficients (r^2) were 0.99 (cysts) and 0.98 (vegetative forms).

Analytical Specificity: Figure 2B shows that none of the tested pathogens' DNA interfered with the MAB-PCR, confirming the good specificity of the MAB-PCR.

Figure 2C shows the low rate of the *intra-assay variance* and *inter-assay variance*. The variation coefficient of Cq ranged from 0.61% to 3.74% for intra-assay variance and from 0.58% to 1.72% for inter-assay variance. Special attention was paid to the fact that for all amounts of human DNA, amplification of one *Acanthamoeba* genome displayed a lower Cq than the beta-globin PCR (data not shown). Clinical in-house isolates (genotypes T4, T3, and T6) were tested: the optimized procedure of the MAB-PCR allowed a correct amplification of each strain.

A



B

	DNA Concentrations (ng/µL)	Cq
S. aureus	66,1	>45
S. epidermidis	78,8	>45
P. aeruginosa	107,9	>45
H. influenzae	113,1	>45
S. pneumoniae	57,1	>45
S. marcescens	93,8	>45
Corynebacterium sp	126,7	>45
P. acnes	8,3	>45
C. albicans	32,4	>45
C. parapsilosis	55,3	>45
A. fumigatus	55,2	>45
Fusarium sp	66,4	>45

C

	Intra-assay variance			Inter-assay variance		
	n	Mean Cq (± SD)	CV (%)	n	Mean Cq (± SD)	CV (%)
1 Cyst + 50 human cells	5	33.8 (± 0.31)	0.90	10	34.04 (± 0.59)	1.72
10 Cysts + 50 human cells	5	30.78 (± 0.36)	1.16	10	30.52 (± 0.18)	0.58
100 Cysts + 50 human cells	5	27.36 (± 0.17)	0.61	10	27.13 (± 0.24)	0.89
1000 Cysts + 50 human cells	5	24.87 (± 0.93)	3.74	10	23.29 (± 0.18)	0.78

n: number of repetitions; SD: standard deviation; Cq: quantification cycle; CV: coefficient of variation

Figure 2. Parameters for the validation of the MAB-PCR assay. (**A**) Linear dynamic range (target *Acanthamoeba* spp.). (**B**) Specificity of the MAB-PCR. (**C**) Evaluation of the intra-assay variance and inter-assay variance of *Acanthamoeba* amplification in the MAB-PCR.

Search for Potential PCR Inhibitors

Figure 3 shows the Cq obtained with the MAB-PCR for one cyst (Fig. 3A) or 100 HFF cells (Fig. 3B) added to 8 µL of different topical eye drops. *Acanthamoeba* PCR and beta-globin PCR were all positive; no solution completely inhibited the PCR reaction. Still, for the *Acanthamoeba* PCR reaction, the eye drops considered partial inhibitors (Cq > mean Cq + 2 SD) were fluorescein, picloxydine, tropicamide, amphotericin B, PolyHexaMéthylèneBiguanide, and ticarcillin. For the beta-globin PCR reaction, picloxydine and PHMB were considered partial inhibitors.

Corneal Scraping Samples

Twenty-eight corneal scraping samples were tested with the MAB-PCR. Table 3 details the results and interpretation of MAB-PCR, the direct exam and the culture for amoeba, available microbiological results, and final diagnosis. Twenty-one samples showed a positive result for the beta-globin target, confirming the presence of scraping material on the scalpel. Seven samples failed to amplify the beta-globin sequence, suggesting that the amount of DNA material was too low to detect the *Acanthamoeba* target. None of the samples tested contained potential inhibitor. On the 21 interpretable results, 18 samples showed a negative result for the *Acanthamoeba* target and 3 samples showed a positive result for the *Acanthamoeba* target. Corneal scraping 4 and 22 were genotyped as T4. DNA extraction volume of the corneal scraping 7 appeared to be insufficient to allow genotyping.

These three patients wore contact lenses. Two patients had a corneal abscess (< 1 mm) and were given hexamidine treatment. Favorable evolution with no loss of visual acuity was noticed after 1 month. One patient presented pseudo-dendritic keratitis; initial loss of visual acuity was reduced under hexamidine treatment (still ongoing).

Discussion

The risk factors for AK have been partially identified and are mostly related to contact lens wear.[22] In our patient series, all patients diagnosed with AK were contact lens users. Nevertheless, recent outbreaks of AK have been described after the distribution of multipurpose contact lens solutions with insufficient anti-*Acanthamoeba* efficacy.[4] In France, this risk factor has not been described, but can also be a potential source of contamination. Although AK is still rare, the growing tendency of this highly morbid infection will certainly be a public health concern in the near future. Rapid and reliable diagnosis of AK is needed for both patients and surveillance of risk factors. The aim of this study was to improve the molecular diagnosis of *Acanthamoeba* keratitis. We confirm the effective detection of *Acanthamoeba* DNA using previously described primers and probes[9] but adapted the PCR protocol to allow a concomitant amplification of human DNA, thus informing clinicians about the scraping quality. This improved tool highly limits the risk of false-negative diagnoses, which misleads ophthalmologists and contributes to inaccurate management of the infection.

The DNA extraction step was optimized to ensure systematic amplification of the lowest concentration of *Acanthamoeba* sp. that can be found in a sample (i.e., a single isolated cyst). In our hands, the extraction procedure combining thermal-shock pretreatment with a manual DNA extraction procedure

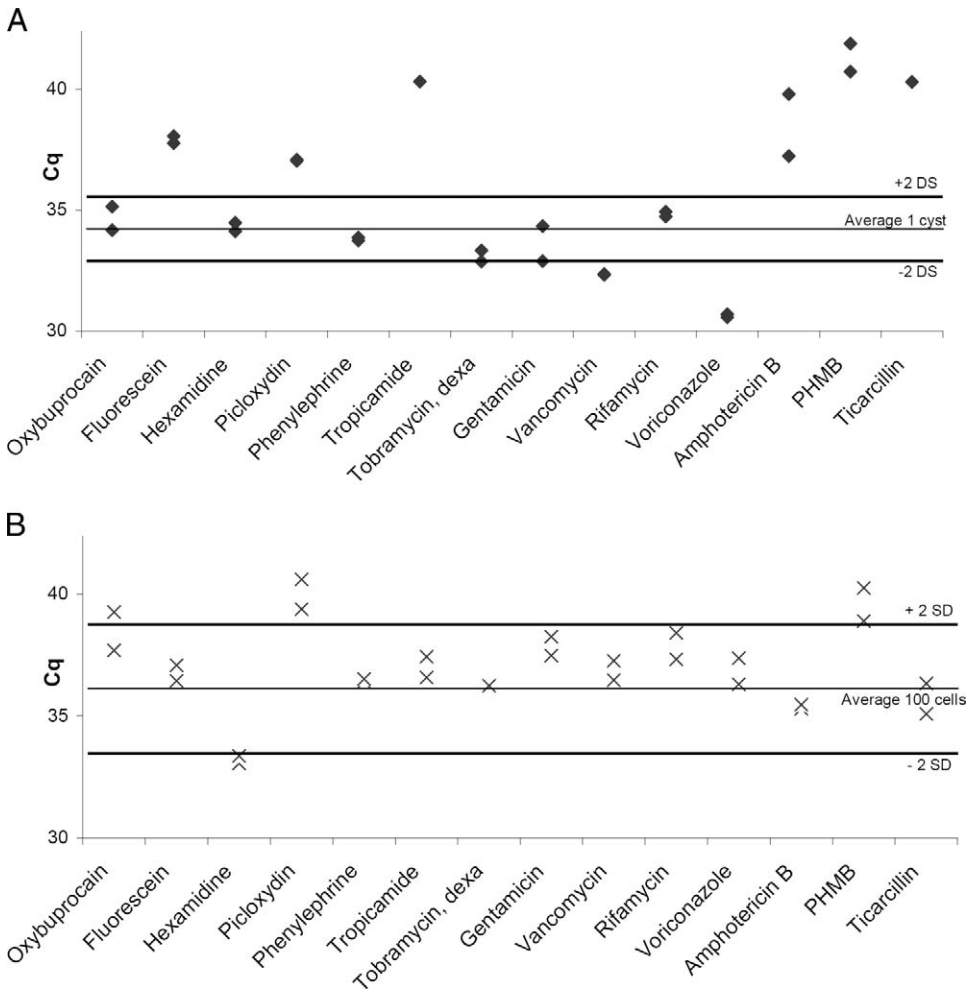


Figure 3. Search for PCR inhibitors: Cq for one cyst (**A**) or 100 HFF cells (**B**) with different topical eye drops (duplicates). Partial inhibition was considered when Cq with the topical drops was greater than the mean Cq + 2 SD.

appeared significantly superior to chemical lysis procedures and the EZ1-Biorobot automated extraction protocol. Our whole assay (combined pretreatment, extraction, and PCR) allowed us to amplify 30 (97%) of 31 isolated cysts (0.2 cyst/PCR tube). This extraction procedure seems to be even more efficient than the one previously described, using Proteinase K and MagnaPure kit (Roche, Penzberg, Germany) extraction, which allowed a DNA amplification in 33% of cases for 0.15 cyst/tube (same genotype),[13] confirming that combination of extraction procedure and amplification protocol is crucial for PCR assay efficiency. In another study, Thompson et al.[9] showed that a number of eye drops could inhibit their PCR reaction. The present result also indicated the potential inhibiting power of PHMB, but at a high concentration, which is probably never reached in an actual scraping sample. In this study, eye drops did not appear to be a real problem for the amplification of the two targets. This discrepancy is probably attributable to the different extraction procedures and internal controls (*Acanthamoeba* cysts versus target DNA). Nevertheless, caution should be exercised and gentle but abundant washing of the cornea is warranted before sampling, especially if ophthalmologists use fluorescein for clinical diagnosis, because fluorescence can interfere with the acquisition system of real-time PCR assays.

In the PCR design used herein, the already described *Acanthamoeba* target[9] was associated with the detection of the beta-globin human gene. The concomitant amplification of this sequence guarantees the presence of scraping material. Testing a prospective series of patients with infectious keratitis, this study showed that seven samples (25%) were not amplified for human DNA, and it was impossible to confirm that the absence of the *Acanthamoeba* amplification was not the consequence of insufficient scraping material. If AK suspicion is high, a new sampling is requested. These results highlight the importance of high-quality sampling to allow PCR interpretation.

MAB-PCR performance was evaluated and it was demonstrated that the procedure displayed high sensitivity, specificity, efficacy, repeatability, and reproducibility. Finally, in a one-step procedure (the same PCR mix), reliable results were produced on the presence or the absence of *Acanthamoeba* DNA, as well as on the quality of the scraping and the presence of inhibitors. The entire procedure, including extraction and amplification, requires 3.5 hours, thus allowing clinicians to adapt their treatment very rapidly with a potential positive effect on the final prognosis.[23]

Because the *Acanthamoeba* genome is highly variable, the ability of the PCR procedure to detect a broad range of genotypes potentially implicated in AK is a concern. The recently described real-time fast duplex TagMan PCR has been shown to detect a wide range of *Acanthamoeba* strains available from the American Type Culture Collection.[11] Here, the primers and probes chosen also tested positively on almost all genotypes implicated in AK, mainly T4, T3, T6, and

Table 3. Results and Interpretation of MAB-PCR, the Direct Exam, and Culture for Free-Living Amoeba and Other Microbiological Results Available, and the Final Diagnosis

	***Acanthamoeba* spp.**								
	MAB-PCR						**Microbiology**		
	Cq Aca	**Cq BG**	**AI**	**Conclusion***	**MGG**	**Cult**	**Bacteriology**	**PCR HSV**	**Final Diagnosis**
1	>45	35.4	AAI	N	ND	ND	*S. epidermidis*	ND	*S. epidermidis* corneal abscess
2	>45	28	AAI	N	N	N	SC	N	Undetermined etiology
3	>45	32.6	AAI	N	N	N	*Stenotrophomonas maltophilia* *Bacillus cereus*	N	*S. maltophilia* and *B. cereus* corneal abscess
4	**30.4**	**37.6**	**AAI**	**P**	**N**	**N**	**SC**	**ND**	***Acanthamoeba* keratitis**
5	>45	28.8	AAI	N	N	N	*P. aeruginosa* *S. aureus*	ND	*P. aeruginosa* conjunctivitis
6	>45	>45	AAI	NC	N	N	SC	N	Undetermined etiology
7	**38.5**	**39**	**AAI**	**P**	**N**	**N**	**SC**	**N**	***Acanthamoeba* keratitis**
8	>45	34.7	AAI	N	N	N	*P. aeruginosa*	N	*P. aeruginosa* corneal abscess
9	>45	33	AAI	N	N	N	SC	N	Undetermined etiology
10	>45	37.6	AAI	N	N	N	SC	ND	Undetermined etiology
11	>45	28.5	AAI	N	N	N	*Moraxella* spp.	N	*Moraxella* sp. corneal abscess
12	>45	44.8	AAI	NC	N	N	SC	N	Undetermined etiology
13	>45	>45	AAI	NC	N	N	SC	P	Herpes simplex keratitis
14	>45	25.4	AAI	N	N	ND	SC	N	Undetermined etiology
15	>45	42.5	AAI	N	ND	ND	SC	N	Undetermined etiology
16	>45	32.2	AAI	N	N	N	*S. aureus*	N	*S. aureus* corneal abscess
17	>45	35	AAI	N	ND	ND	SC	N	Undetermined etiology
18	>45	41	AAI	N	ND	ND	SC	N	Herpetic disciform keratitis
19	>45	>45	AAI	NC	ND	N	ND	ND	Recurrent keratitis
20	>45	39	AAI	N	N	N	SC	N	Undertemined etiology
21	>45	>45	AAI	NC	N	N	ND	ND	Undetermined etiology
22	**29.9**	**30.1**	**AAI**	**P**	**ND**	**ND**	**SC**	**ND**	***Acanthamoeba* keratitis**
23	>45	40	AAI	N	N	ND	SC	N	Undetermined etiology
24	>45	>45	AAI	NC	ND	ND	SC	ND	Undetermined etiology
25	>45	28.3	AAI	N	N	N	SC	N	Undetermined etiology
26	>45	40	AAI	N	ND	ND	ND	ND	Undetermined etiology
27	>45	26.3	AAI	N	N	N	*P. aeruginosa* *Klebsiella* sp.	N	*P. aeruginosa* and *Klebsiella* sp. severe corneal abscess
28	>45	>45	AAI	NC	ND	ND	SC	ND	Undetermined etiology

* N, negative: Absence of *Acanthamoeba* spp. DNA, presence of scraping specimen, no amplification inhibitors; P, positive: Presence of *Acanthamoeba* spp. DNA in corneal scraping, result in favor of *Acanthamoeba* keratitis; NC, not contributive: absence of human DNA amplification, lack of scraping material, absence of inhibitors; AAI, absence of amplification inhibitors; Cq, quantitative cycle; MGG, May-Grunwald-Giemsa staining; HSV, herpes simplex virus; SC, sterile culture.

T11, and to a much lesser extent T7, T10, T1, and T14.[9,12,24] One strain belonging to genotype T5 was not amplified with these primer sets.[11] Hence, BLAST on other strains belonging to the T5 genotype showed perfect hybridization with the Acant primers and probe sequences (Supplementary Material, available at http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-8587/-/DCSupplemental). The variability of the genome structure makes it difficult to test all the species and genotypes of *Acanthamoeba*. Moreover, even between isolates of a same genotype there is up to 5% sequence difference[25] between isolates of a same genotype. In addition, knowing that one species can belong to different genotypes, no conclusion can be drawn on one isolated strain. Moreover, comparative studies on efficiency of different real-time PCR assays on real sampling can be contradictory, maybe owing to the use of different extraction methods.[9,26] To date, all accessible data reported that the Acant sequences could be adapted to diagnose AK. The combination of several primer sets can also be considered to increase the sensitivity of AK diagnosis.[8]

Most of the already-described PCR assays show good sensitivity and specificity for AK diagnosis, but none of them aim to evaluate sample quality concomitantly, which is clearly a limiting factor for sensitive diagnosis. In the present series, over a 3-month period, 10.7% of the scrapings were positive for *Acanthamoeba* DNA, but displayed negative direct examination and culture results (available for two of the three samplings). These results were not considered false-positive because genotyping using other primers confirmed the presence of *Acanthamoeba* DNA and because the clinical outcome was favorable with specific treatment. This confirms that, as shown with other PCR methods,[18,26] the MAB-PCR outperforms the usual "gold standard" for AK diagnosis.

Another essential question has recently been raised concerning the length of AK treatment.[27] Similar to culture, PCR follow-up of corneal scraping could be tested to evaluate the clearance of the pathogen, even if detection of *Acanthamoeba* DNA could occur in the presence of dead microorganisms. Other AK diagnostic complementary tools, such as in vivo confocal microscopy,[28–30] could also be used to monitor treatment response, but this remains to be evaluated. Association with an efficient molecular diagnostic could allow the evaluation for this noninvasive diagnostic of AK.

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