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**Development, optimization, and validation of a multiplex real-time PCR assay on the  
BDMAX platform for routine diagnosis of *Acanthamoeba* keratitis**

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## **Abstract**

The reported number of cases of *Acanthamoeba* keratitis (AK) is continually increasing. Molecular diagnosis has become the first choice of ophthalmologists for identifying and confirming this clinically problematic diagnosis. However, in-house molecular procedures are time-consuming and may not be compatible with the urgency of the situation. In this study, we adapted a previous in-house AK-PCR, on the BDMAX, a full-integrated automated platform for molecular biology (Becton Dickinson, Heidelberg, Germany), for the rapid routine diagnosis of *Acanthamoeba* keratitis. We compared different protocols to optimize DNA extraction from *Acanthamoeba* cysts. We evaluated the analytical parameters of the AK-BDMAX-PCR. Thirty-two samples were simultaneously tested with the AK-BDMAX PCR and the original AK-PCR from which it was developed. A thermal-shock pretreatment protocol was validated. The analytical parameters were similar to those obtained with the previous in-house AK-PCR method. We then assessed the performance of the AK-BDMAX PCR for routine testing on 40 clinical samples, mostly corneal scrapings. Frozen ready-to-use in-house PCR premixes were stable over eight months. Overall, 34 (85%) of the 40 clinical samples were considered to be true negatives, four (10%) were considered to correspond to probable AK and two (5%) were considered to correspond to possible AK.

This newly developed AK-BDMAX-PCR is reliable, rapid and efficient, and should facilitate *Acanthamoeba* keratitis diagnosis.

# INTRODUCTION

Amebic keratitis (AK) is a rare but potentially sight-threatening infection, caused by free-living amoebae of the genus *Acanthamoeba*. These amoeba are naturally present in tap water and freshwater bodies, and are emerging pathogens in ophthalmology, due particularly to the increase in contact lens use, greater awareness among ophthalmologists and improvements in diagnostic technologies<sup>1-4</sup>.

The wearing of contact lenses is a risk factor for corneal infections, and contact lens users account for 85% of all cases of amebic keratitis in Western countries<sup>5</sup>. Previous studies have shown that lens care guidelines were almost never followed correctly by patients with amebic keratitis<sup>6,7</sup>. The clinical diagnosis of AK can be challenging, and prognosis is directly related to the promptness of diagnosis. A method for rapid and specific diagnosis is, therefore, required<sup>4,8,9</sup>.

Previous procedures for the diagnosis of AK were based on classical approaches (microscopy, *in vitro* culture), which were used at our institute (Grenoble Alpes University Hospital, France) together with a previously developed real-time PCR on a Smart Cycler (SC) 2.0 PCR robot (Cepheid, California, USA)<sup>10</sup>. This AK-PCR was based on amplification of the *Acanthamoeba RNA18S*, with concomitant evaluation of the quality of the corneal scraping through amplification of the human beta globin gene (*HBB* gene)<sup>10</sup>. Prior treatment of the sample by thermal shock was required for the optimal extraction of *Acanthamoeba* cysts, for manual DNA extraction<sup>10</sup>. In this previous AK-PCR, the PCR mix had to be prepared before each PCR run. Thus, the entire procedure, from sample to PCR results, was time-consuming, taking about a week at our institute, which was not satisfactory given the need for early diagnosis and specific treatment in cases of confirmed AK.

Laboratory workflows are currently being redesigned, with the development of benchtop and random-access platforms allowing more flexible and efficient molecular diagnosis. These

sample-to-answer platforms provide results much more rapidly. The BDMAX™ system (Becton Dickinson, Heidelberg, Germany) is a fully integrated automated platform for molecular testing. It is of particular interest because it allows laboratories to perform both ready-to-use and user-developed assays on an “open” instrument<sup>11–13</sup>. It is also highly suitable for the treatment of single samples, with extraction and amplification achieved in a single run.

The aim of this study was to develop and validate a new AK-PCR on the BDMAX platform, to accelerate the diagnosis of AK.

## **MATERIALS AND METHODS**

### ***Acanthamoeba* strains**

A clinical T4 genotype *Acanthamoeba* strain was used to optimize the DNA extraction process and to validate the PCR assay. The strain was maintained on a non-nutrient agar plate overlaid with a suspension of *Escherichia coli* (ATCC 25922), as previously described<sup>10</sup>. Encystment was morphologically checked daily with an inverted microscope. When the *Acanthamoeba* was totally encysted, usually after four to seven days, the cysts were collected by scraping, suspended in sterile water and counted in a cell-counting chamber. The suspension was then diluted to the desired concentration and into the wells of a 96-well plate (50 µL/well): we checked that the number of cysts per well was as expected, by examination under an inverted microscope, before performing the experiments<sup>10</sup>.

### **Sample pretreatment**

Previous studies have demonstrated the added value of a pretreatment step before the classical extraction procedure<sup>10,14–16</sup>. We evaluated the value of sample pretreatment before DNA extraction on the BDMAX platform. We performed PCR on 49 samples, each containing two to seven cysts; 26 of these samples were subjected to pretreatment and 23 were not. The pretreatment consisted of one freeze-thaw cycles of 3 minutes at 95°C and 3 minutes at -80°C.

### **Primers and probes and composition of the PCR premix**

Primers and hydrolysis probes (targeting both *Acanthamoeba RNAI8S* and human hemoglobin subunit beta (*HBB*) gene fragments) were as described for the initial in-house PCR Thompson *et al.*<sup>10,14</sup>, except that the *Acanthamoeba-RNAI8S* probe was ROX-labeled to limit the risk of fluorescence shading in the BDMAX sample process control (SPC) detection channel. These primers have been shown to amplify most of the *Acanthamoeba* genotypes implicated in corneal disease<sup>10,14,17,18</sup>.

The premix composition and the sequences of the primers and probes are shown in Table 1. Ready-to-use premixes were prepared, aliquoted into single-use conical tubes fitting the BDMAX extraction strip, sealed with the PlateMax sealer (Axygen Scientific, 8 s at 180°C), and stored at -20°C until use.

#### **BDMAX: extraction and PCR procedure**

Samples or *Acanthamoeba* cysts (with or without pretreatment) were homogenized in 500 µL of PBS, transferred into the sample buffer tubes of the BDMAX ExK DNA-1 kit and placed on the unit racks. The sample buffer tubes contained a combination of lytic and extraction reagents for cell lysis, DNA extraction and inhibitor removal, together with the SPC as an internal control of extraction and amplification. This SPC consisted of a fragment of the *Drosophila melanogaster* genome.

Prepared premixes were defrosted at room temperature, and clipped onto the reaction strips with two additional conical tubes containing the BDMAX ExK DNA-1 extraction reagents and the BDMAX™ DNA MMK (SPC) mastermix. This ready-to-use mastermix contains dNTPs, MgCl<sub>2</sub>, the DNA polymerase and primers and probes for amplification of the SPC. The whole DNA extraction procedure was then performed automatically by the platform. The “Master Mix Format” was “Type 2: BD MMK or MMK (SPC) and liquid primers and probes”. ExK DNA-1 default extraction parameters were used, except that duration of the lysis step was increased to 15 min at 70°C. PCR was performed as follows: an initial single “hold” step consisting of heating for 10 min at 98°C, followed by 45 cycles of 9.9 s at 98°C, 15.7 s at 58°C and 18.7 s at

72°C. The detection parameters for the 475/520 (FAM, *HBB* target), 585/630 (ROX, *Acanthamoeba RNA18S* target) and 680/715 (SPC) channels were adjusted as follows: “gain”: 50, “Threshold”: 50 (475/520 and 585/630) or 100 (680/715). Signal intensities below 50 were considered “negative”, and those above 50 were considered “positive”.

The positive control DNA for *Acanthamoeba* was obtained from a suspension of *A. polyphaga* cysts and the positive control for human DNA was extracted from a cell culture of human foreskin fibroblasts (HFFs), as previously described <sup>10</sup>. The number of cells was considered acceptable if Cq *HBB* <39; if Cq *HBB* ≥ 39, we considered that there were too few cells to ensure a reliable enough diagnosis by *Acanthamoeba* PCR. The negative control was sterile saline solution.

### **Evaluation of the performances of the PCR protocol**

Intra- and inter-assay variabilities were evaluated by testing the *Acanthamoeba* DNA positive control at two different concentrations (pure and 1/20 dilution), 7 to 12 times (Table 2). We dispensed 5 µL of positive control solution into the sample buffer tube, which was then subjected to the extraction and amplification procedures on the BDMAX platform. Quantitative threshold values (or quantification cycles: Cq) were then compared. The specificity of the amplification step was demonstrated for our previous in-house AK-PCR <sup>10</sup>, but we nevertheless checked the specificity of the AK-BDMAX technique, using five DNA extracts from microorganisms frequently associated with infectious keratitis (*Staphylococcus aureus*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa*, *Candida parapsilosis* and *Fusarium* spp.). The efficiency, dynamic range and limit of detection of the PCR were assessed with a standard curve obtained with serial dilutions of DNA corresponding to 0.2 to 200 genomes of *Acanthamoeba*. The limit of detection for *Acanthamoeba* cysts (including the extraction step) was also evaluated by testing six solutions, each containing 3 to 10 cysts. These samples were tested on both the SC 2.0 (SC) and BDMAX platforms. Cross-contamination between samples was assessed by alternating positive and negative controls in a single run. Frozen PCR premix

stability was evaluated on the same positive control on days 1, 3, 10, 15, 25, 30 and 240 (8 months).

The AK-BDMAX and AK-SC PCR techniques were compared on 30 samples: 18 positive samples and 12 negative samples. Positive corneal scrapings are rare, and are invasive. We therefore used 18 artificial positive samples obtained by a gentle scraping of culture medium containing encysted *Acanthamoeba* cysts. For each sample, two needles (25G, BD Microlance™3) were used consecutively for the scraping of the culture, and sampling was controlled visually, by microscopy, to optimize reproducibility. For negative samples, we used 12 corneal scrapings from patients who tested negative AK-SC-PCR. The results obtained with this previous PCR test were correlated with those obtained with the AK-BDMAX-PCR performed on the corneal cell smears sent, on a glass slide, for each patient, at the same time as the original sample, for the purposes of direct examination. We checked the concordance of qualitative (positive/negative) and quantitative (Cq value) results.

### **Clinical samples**

The 40 clinical samples (38 corneal scrapings, 1 corneal biopsy specimen and one lens liquid sample) used for AK-BDMAX evaluation were obtained from 40 patients with suspected infectious keratitis requiring deep scraping of the cornea under slit lamp examination. Samples were collected with sterile stainless steel scalpel blades or fine needles. No written consent was required for sampling, because this is the usual procedure for AK diagnosis at our institute, but patients were informed of the corneal scraping procedure and the potential benefits of microbiological diagnosis. For PCR, the scrapings were transferred to the laboratory in a dry condition, in a sterile 1.5 mL microtube. Before the thermal shock pretreatment, we added 500 µL of sterile saline solution to the sample buffer tube (to reach the minimum volume required for the extraction procedure), and 50 µL of this mixture was dispensed into the collection microtube carrying the needle or the scalpel blade such that the end of the instrument was immersed in the solution. The sample was subjected to a freeze-thaw cycle directly in the 1.5



mL collection tube. After pretreatment, the blade or needle was removed from the collection tube, and 50  $\mu$ L of the solution was dispensed into the BDMAX ExK DNA-1 sample buffer tube for the automated extraction procedure. When two scrapings were performed for a given patient, one blade (or needle) was used for PCR and the other was used for conventional culture (non-nutrient agar plates overlaid with a suspension of *Escherichia coli* (ATCC 25922) and incubated at 27°C for up to 21 days). Depending on the clinical condition of the patient, a corneal cell smear was also obtained on a glass slide, for rapid May-Grünwald-Giemsa staining (Kit RAL 555, RAL Diagnostics, Martillac, France) and direct examination. The final diagnosis was as follows: 1- “probable AK”: presence of *Acanthamoeba* (DNA or culture) with a compatible clinical presentation and a favorable course on specific treatment with no other associated diagnosis, or 2- “possible AK”: presence of *Acanthamoeba* DNA with a compatible clinical presentation and a favorable course on nonspecific antiseptic treatment or the presence of another infectious agent.

## RESULTS

### Sample pretreatment

We assessed the usefulness of sample pretreatment, by performing the AK-BDMAX technique on 49 samples (each containing 2 to 7 *Acanthamoeba* cysts), 26 of which were pretreated and 23 of which were not. *Acanthamoeba* DNA was detected in 77% of the pretreated samples and 61% of the non-pretreated samples (Figure 1A and Table 3). This difference was not statistically significant ( $\chi^2 = 1.48$ , ns), but the rate of positive results was higher after freeze-thaw pretreatment. The mean Cq for pretreated and non-pretreated positive samples was 33.03 ( $\pm 1.36$ ) and 32.52 ( $\pm 2.33$ ), respectively ( $t$ -test = 0.7, ns).

### Performance of the AK-BDMAX method

Intra-assay and inter-assay variabilities were low for the AK-BDMAX method. The means, standard deviations and the coefficient of variation of Cq are shown in Table 2. No cross-contamination between samples was observed (data not shown). As expected, no cross-reaction

was detected with *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa*, *Candida parapsilosis* or *Fusarium* spp. Over an eight-month period, the same *Acanthamoeba* positive control was tested seven times with the same ready-to-use frozen premix containing primers and probes for *Acanthamoeba* and human DNA detection. The mean Cq was 28.13 ( $\pm$  0.95), with a coefficient of variation of 3.37%. The ready-to-use in-house premix was, therefore, stable over at least eight months at -20°C. In analyses of PCR efficiency, the dynamic range of the assay resulted in a standard curve correlation coefficient ( $r^2$ ) of 0.998, with a limit of detection of 0.2 *Acanthamoeba* genome equivalents in the sample tube (Figure 1B). The cyst detection threshold was also assessed by comparing samples with different cyst concentrations tested with the AK-BDMAX and AK-SC PCR assays. The results are given in Table 4. All samples (6/6) were positive, so the detection threshold on BDMAX is at least four cysts in the sample tube, corresponding to approximately 0.4 genome equivalents in the final PCR chamber (Table 4).

### **Comparison with the previous in-house PCR protocol**

Eighteen positive samples (scrapings from positive cultures) and 12 negative samples (smears or needles from corneal scrapings) were analyzed, to compare the AK-SC and AK-BDMAX assays (Table 5).

All positive samples tested positive for the *Acanthamoeba* spp. target with both the AK-SC and AK-BDMAX assays, resulting in a qualitative concordance of 100% between these two techniques. The mean and median Cq were 35.8 and 34.9 (range: 29.5-44) for AK-BDMAX-PCR and 35.7 and 34.9 (range: 31.3-43.6) for AK-SC PCR.

Similarly, all negative samples tested negative for the *Acanthamoeba* spp. target with both techniques. We checked that the results from the needles could be reliably compared with the results from the corresponding corneal smears by concomitantly evaluating sample cellularity by amplifying a fragment of the *HBB* gene. The mean and median Cq for the *HBB* target were

similar for both types of samples: 32.8 and 32.7 (range: 27.5-39) for AK-BDMAX-PCR (smears) and 32.6 and 33 (range: 27-38) for AK-SC PCR (needles).

### **Evaluation of the AK-BDMAX technique for routine use**

We obtained 40 clinical samples (38 corneal scrapings, 1 corneal biopsy specimen and one lens liquid sample) from 40 patients with suspected infectious keratitis for evaluation of the AK-BDMAX technique in routine use (Table 6). None of these samples contained potential PCR inhibitors. For five samples (12.5%), we were unable to amplify significant amounts of the *HBB* sequence ( $Cq \geq 39$ ), suggesting that there was probably too little corneal DNA present to detect *Acanthamoeba*. Thus, from the corresponding AK-BDMAX-PCR results, we were unable to determine whether *Acanthamoeba* was present or absent in the scraping. Ophthalmologists were advised to send a new sample only if symptoms persisted, and if no other possible cause could be identified. We obtained 35 interpretable results: 29 (72.5%) samples tested negative for the *Acanthamoeba* target and six samples (15%) tested positive for *Acanthamoeba* DNA. Based on the clinical context and the microbiological results detailed in Table 6, AK was considered ‘probable’ in patients 19, 33, 35 and 40, and ‘possible’ in patients 10 and 32. Neither direct examination (DE) nor culture was positive for *Acanthamoeba* for any of these patients.

## **DISCUSSION**

*Acanthamoeba* has become a significant etiology of infectious keratitis, due particularly to increases in contact lens use<sup>1-3</sup>. In this context, the rapid detection of this emerging pathogen is required to initiate early targeted treatment, avoiding the need for penetrating keratoplasty<sup>19</sup>. In recent years, PCR has become a key tool for AK diagnosis, as for other ocular infectious diseases, and has been shown to outperform conventional non-molecular tests in terms of sensitivity and specificity<sup>15,20,21</sup>. We describe here the adaptation of a previously developed in-house AK-PCR on the BDMAX molecular platform, to facilitate prompt AK diagnosis<sup>10</sup>. The performance of the adapted AK-BDMAX PCR met with the necessary standards for qPCR applications. This AK-BDMAX-PCR had low intra-assay and inter-assay variabilities, as for

our previous technique <sup>10</sup>. Specificity for *Acanthamoeba* detection was conserved, as no cross-reaction with other microorganisms was observed. We also detected no inter-sample contamination between adjacent reagent strips. In addition, the presence of the SPC (internal control) made it easy to identify technical issues or to detect the presence of PCR inhibitors. Indeed, the SPC was present in the sample extraction tube, so its DNA was extracted and amplified at the same time as the *Acant-RNA18S* and *HBB* gene fragments, making control of the entire process possible on the BDMAX platform. Based on these results, we concluded that the performance of the AK-BDMAX-PCR was compatible with its integration into the set of techniques used at our institute.

We evaluated the added value of pretreatment before automated DNA extraction, because the cell wall of *Acanthamoeba* cysts can be hard to disrupt and other studies have demonstrated the utility of this pretreatment procedure for other tests <sup>10,14,16</sup>. Thermal shock is a mechanical pretreatment that weakens the cyst cell wall, facilitating subsequent DNA extraction. We initially used three freeze-thaw cycles (100°C/-80°C), but, based on published findings and our own initial results, we decreased the pretreatment to a single freeze-thaw cycle <sup>14</sup>. The results were not statistically significant, but were in favor of a pretreatment of the samples before extraction (positivity rate of 77% vs. 61%, ns). Several other protocols for cell wall disruption have been described, including bead extraction methods, which also seem to be an efficient way to optimize DNA extraction <sup>22</sup>.

Molecular biology platforms are continually being adapted to the needs of users. Ready-to-use kits and random access platforms are currently being expanded in microbiological laboratories. Nevertheless, despite the threat to sight that it represents, necessitating rapid, specific treatment, AK has not really attracted the attention of *in vitro* diagnostic companies, and, to our knowledge, no commercial kit has yet been developed or correctly evaluated for the *in vitro* diagnosis of AK. Furthermore, the assays currently available on the market are labeled 'research use only' and cannot be used to evaluate scraping cellularity, a crucial parameter, in our opinion, for accurate final AK-PCR interpretation. Indeed, corneal scraping can result in very

few corneal cells being obtained, which may limit sensitivity. The suitability of the scraping for testing is also highly informative for ophthalmologists. Finally, the BDMAX platform allows the use of premixes prepared in advance and frozen in single sealed tubes, which is convenient for rapid individual testing. Thus, although the entire process (i.e. pretreatment, extraction, amplification) still takes two hours, this includes only 10 minutes of effective technician working time for pretreatment and reaction strip preparation. The BDMAX platform can be used to adapt previously developed in-house PCRs into a fully integrated and automated on-demand molecular diagnostic system, and it has already been used and validated for many other microbiological targets <sup>11,12,23,24</sup>. The linear dynamic range revealed a theoretical limit of detection of 0.2 genomes/PCR, but the automated extraction protocol of the BDMAX is performed on 950/1250 µL from the BDMax sample tube so, if there is only one cyst in the extraction tube, there is a small probability (1 out of 4) of the cyst not being extracted. Thus, although AK-BDMAX can detect one or two cysts in the sample tube, we think that at least three or four cysts would be a more “comfortable” input to ensure maximum sensitivity of the test. This issue also justifies concomitant control of the amount of human DNA present in the sample, based on the assumption that the more scraping material obtained, the more *Acanthamoeba* cysts are likely to be retrieved. In addition, ophthalmologists are aware that a negative scraping result does not formally eliminate *Acanthamoeba* keratitis, and in cases of strong clinical suspicion, a corneal biopsy is generally performed, resulting in the retrieval of larger numbers of cysts in cases of *Acanthamoeba* keratitis.

In terms of its clinical appearance, AK can easily be confused with other etiologies of infectious keratitis. This often results in late diagnosis, with many patients already having undergone days or weeks of antimicrobial treatment, which can alter culture or AK-PCR results <sup>25,26</sup>. It can therefore be difficult to obtain a definitive biological diagnosis in the absence of a corneal biopsy, because a weak positive PCR result may be difficult to interpret. Furthermore, AK is often polymicrobial, and *Acanthamoeba* spp. are susceptible to some antiseptics or antifungal agents, which may be misleading, resulting in an erroneous final diagnosis <sup>4,27,28</sup>. During the

study period, six (15%) of the 40 patients presented with positive AK-PCR results. Four (10%) were considered by the ophthalmologist to have probable AK, but in two patients (5%) the diagnosis was no firmer than ‘possible AK’. Both patients with ‘possible’ AK displayed very weak positive DNA amplification (Cq = 44), possibly because *Acanthamoeba* is a free-living microorganism that may be commensal in the normal eye, but is also found in various eye diseases, in association with other pathogens<sup>28</sup>. A recent study suggested that the incidence of *Acanthamoeba* spp. DNA in healthy contact lens-wearers is high, although only 1.1% gave positive culture results<sup>29</sup>. ‘Free’ *Acanthamoeba* DNA can clearly contaminate the eye surface in some cases. Given these elements, it remains necessary to determine the precise medical significance of weakly positive PCR results and their implication for the management of the patient. Although the results show that the AK-BMAX PCR is a step forward in making diagnostics easier and more rapid, this study presents some limitations, especially when considering the nature of positive samples, mostly artificial ones. Only a low number of clinical samples were included, as AK samples of confirmed AK cases are difficult to obtain. In addition, inter-laboratory exchange controls are also based on artificial samples. Therefore, as for other *Acanthamoeba* PCR techniques, it is still difficult to accurately establish the exact sensitivity and specificity of the AK-BDMAX PCR in clinical settings.

## **CONCLUSION**

*Acanthamoeba* keratitis is a rare, sight-threatening eye infection requiring prompt, specific treatment. We successfully adapted a real-time PCR targeting *Acanthamoeba* spp. DNA for use on a fully automated PCR platform suitable for use in routine diagnosis, which will improve the everyday management of infectious keratitis at our institute.

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# TABLES

Table 1: AK-BDMAX PCR premix composition

Premix components - concentration	Volume/reaction (μL)	Sequences
Sterile water	7.5	
BDMAX primer and probe diluent	2	
Acant <i>RNA18S</i> - probe - 20 μM	0.6	5'-[ROX]TG[+C]CA[+C]CGAA[+T]A[+C]A [BHQ2]-3'
Acant <i>RNA18S</i> - F900 primer - 20 μM	0.5	5'-CCCAGATCGTTTACCGTGAA--3'
Acant <i>RNA18S</i> - R1100 primer - 20 μM	0.5	5'-TAAATATTAATGCCCCCAACTATCC-3'
<i>HBB</i> - probe - 20 μM	0.4	5'-[FAM]TCCTGAGACTTCCACACTGATGC[BHQ1]-3'
<i>HBB</i> - F primer - 20 μM	0.5	5'-TGAGTCTATGGGACGCTTGA-3'
<i>HBB</i> - R primer - 20 μM	0.5	5'-AAAAATTGCGGAGAAGAAAAA-3'
<b>Total (μL)</b>	12.5	

HBB: hemoglobin subunit beta

Table 2: Intraassay and interassay variabilities of *Acanthamoeba* amplification

	<b>Sample</b>	<b><i>n</i></b>	<b>Mean Cq (<math>\pm</math>SD)</b>	<b>CV (%)</b>
Intra-assay variability	Positive control	7	29.30 (0.51)	1.73
	1/20-diluted positive control	7	32.99 (0.60)	1.81
Inter-assay variability	Positive control	12	28.91 (0.98)	3.38

Cq: quantification cycle ; SD: standard deviation ; CV: Coefficient of variance.

Table 3: Comparison of the results obtained with and without pretreatment in the AK-BDMAX PCR

	<b>Thermal shock</b>	<b>No pretreatment</b>
<i>N</i> positive samples/ <i>N</i> samples tested	20/26	14/23
Positivity rate (mean Cq±SD)	77% (33.03±1.36)	61% (32.52±2.33)

Cq: quantification cycle ; SD: standard deviation

Table 4: Threshold for cyst detection

Number of cysts		PCR result		Cq	
AK-BDMAX	AK-SC	BDMAX	AK-SC	AK-BDMAX	AK-SC
4	3	+	+	37.8	40.3
5	6	+	+	33.5	34.8
10	8	+	+	29.6	36

Table 5: Comparison of AK-BDMAX PCR with our previous in-house AK-SC PCR protocol

<i>n</i>	Sample type		PCR result		Mean/median Cq (range)		Concordance
			AK-BDMAX	AK-SC	AK-BDMAX	AK-SC	
18	Artificial	AK	P (18)	P(18)	35.8/34.9 (29.5-44)	35.7/34.9 (31.3-43.6)	100% (18/18)
14	Corneal scraping	AK	N (12)	N (12)	>45	>45	100% (12/12)
	Smear	HBB	P (12)	P (12)	32.8/32.7 (27.5-39)	32.6/33.0 (27-38)	100% (12/12)

N: negative; P: positive; AK: *Acanthamoeba* keratitis ; HBB: hemoglobin subunit beta



Table 6: Clinical samples: results and interpretation of AK-BDMAX PCR, direct examination and culture for free-living amoebae, other microbiological results available, and final diagnosis

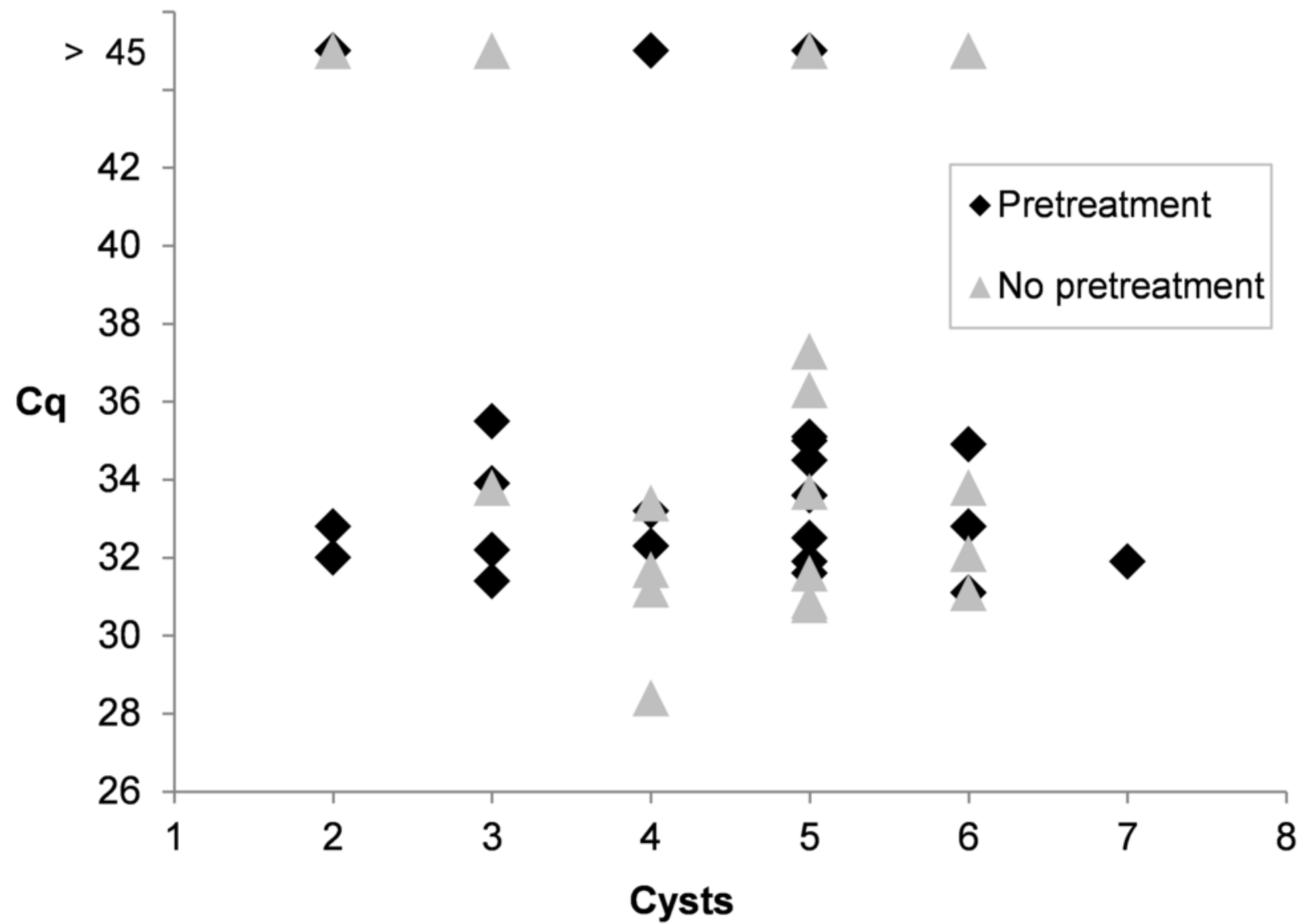
<i>Acanthamoeba</i> spp.							Microbiology			Final diagnosis for AK
Patient	Cq Acant	Cq HBB	SPC	PCR interpretation*	MG G	Cult	Bacteriology	Virology	Mycology	
1	>45	25	AAI	N	N	N	SC		SC	Other diagnosis
2	>45	39	AAI	NC		N				Other diagnosis
3	>45	36	AAI	N			SC	N		Other diagnosis
4	>45	35	AAI	N		N	SC	N		Other diagnosis
5	>45	31	AAI	N		N	<i>Streptococcus sanguinis</i>	HSV1	SC	Other diagnosis
6	>45	40	AAI	N (Lens liquid)		N	SC			Other diagnosis
7	>45	35	AAI	N		N		HSV1 (detectable)	SC	Other diagnosis
8	>45	40	AAI	NC		N		N	SC	Other diagnosis
9	>45	33	AAI	N			SC	N	SC	Other diagnosis
10	44	38	AAI	P			SC	N	SC	Possible AK
11	>45	39	AAI	NC			SC	N	SC	Other diagnosis
12	>45	35	AAI	N			SC	N	SC	Other diagnosis
13	>45	34	AAI	N			SC	N		Other diagnosis
14	>45	40	AAI	NC		N	<i>Staphylococcus aureus (lens)</i>	N	SC	Other diagnosis
15	>45	36	AAI	N		N	SC	N	SC	Other diagnosis
16	>45	33	AAI	N		N	SC	N	SC	Other diagnosis
17	>45	37	AAI	N		N		VZV (traces)	SC	Other diagnosis
18	>45	34	AAI	N		N	SC		SC	Other diagnosis
19	38	37	AAI	P		N	SC			Probable AK
20	>45	32	AAI	N		N	SC	N	SC	Other diagnosis
21	>45	29	AAI	N		N	SC	N		Other diagnosis
22	>45	36	AAI	N		N	SC	N		Other diagnosis
23	>45	34	AAI	N		N	<i>Pseudomonas aeruginosa</i>	N	SC	Other diagnosis
24	>45	39	AAI	NC		N	SC	N	SC	Other diagnosis
25	>45	35	AAI	N		N	SC	N	SC	Other diagnosis
26	>45	34	AAI	N		N	SC	N	SC	Other diagnosis
27	>45	35	AAI	N		N		VZV (high viral)		Other diagnosis

								load)			
28	>45	34	AAI	N		N		SC	N	Other diagnosis	
29	>45	31	AAI	N		N		SC	N	SC	Other diagnosis
30	>45	34	AAI	N			N		N		Other diagnosis
31	>45	30	AAI	N		N			N	SC	Other diagnosis
32	44	33	AAI	P				SC	HSV1 (detectable)	SC	Possible AK
33	37.4	38	AAI	P		N		SC	N	SC	Probable AK
34	>45	37	AAI	N		N		SC	HSV1 (high viral load)	SC	
35	36	35	AAI	P		N	N				Probable AK
36	>45	37	AAI	N				SC	N	<i>Fusarium</i> sp. (lens)	Other diagnosis
37	>45	38	AAI	N		N	N	SC	N	SC	Other diagnosis
38	>45	34	AAI	N				SC	HSV1 (high viral load)	SC	Other diagnosis
39	>45	32	AAI	N		N	N	SC	N	SC	Other diagnosis
40	40	35	AAI	P		N	N	SC	N	SC	Probable AK

\*AK-BD MAX PCR interpretation: 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 consistent with *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. VZV: varicella-zoster virus. SC = sterile culture. HBB: hemoglobin subunit beta. SPC: Sample process control

Figure 1:

A- Comparison of pretreatment procedures; B: linear dynamic range of the AK-BDMAX PCR

**A****B**