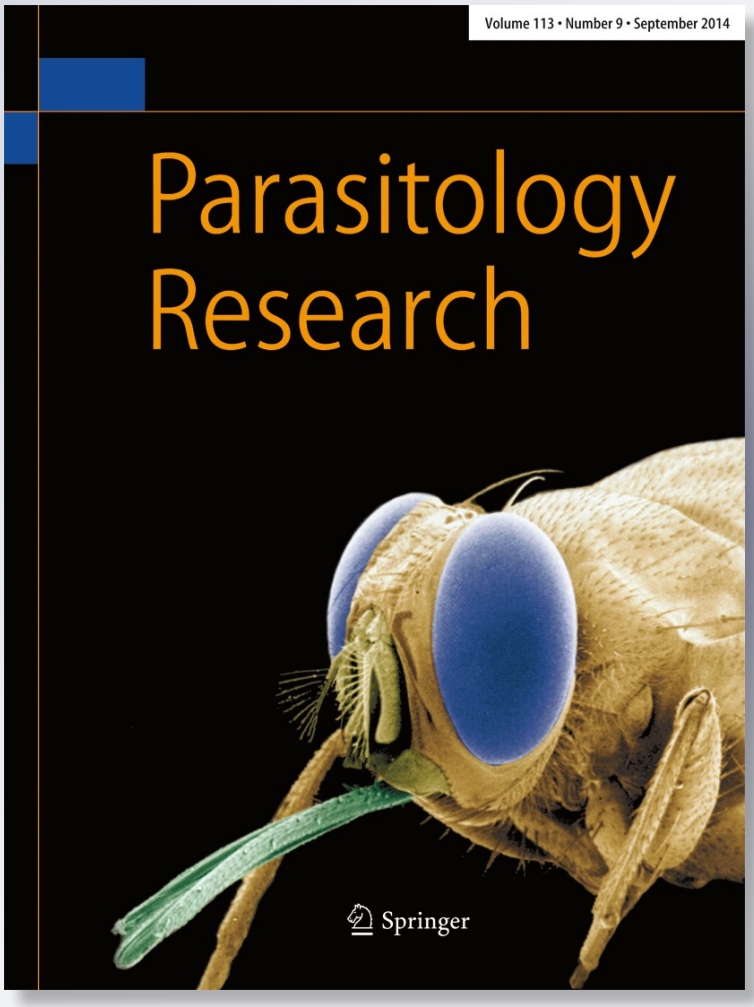
Acanthamoeba *DNA can be directly amplified from corneal scrapings*

Nagwa Mostafa El-Sayed, Mohamed Saad Younis, Azza Mohamed Elhamshary, Amina Ibrahim Abd-Elmaboud & Shereen Magdy Kishik

Parasitology Research
Founded as Zeitschrift für Parasitenkunde

ISSN 0932-0113
Volume 113
Number 9

Parasitol Res (2014) 113:3267-3272
DOI 10.1007/s00436-014-3989-3



Springer

Parasitol Res (2014) 113:3267–3272
DOI 10.1007/s00436-014-3989-3

ORIGINAL PAPER

Acanthamoeba DNA can be directly amplified from corneal scrapings

Nagwa Mostafa El-Sayed · Mohamed Saad Younis · Azza Mohamed Elhamshary · Amina Ibrahim Abd-Elmaboud · Shereen Magdy Kishik

Received: 4 March 2014 / Accepted: 6 June 2014 / Published online: 21 June 2014

Abstract This study evaluated the performance of direct amplification of *Acanthamoeba*-DNA bypassing DNA extraction in the diagnosis of *Acanthamoeba* keratitis in clinically suspected cases in comparison to direct microscopic examination and in vitro culture. Corneal scrapings were collected from 110 patients who were clinically suspected to have *Acanthamoeba* keratitis, 63 contact lens wearers (CLW), and 47 non-contact lens wearers (NCLW). Taken samples were subjected to direct microscopic examination, cultivation onto the non-nutrient agar plate surface seeded with *Escherichia coli*, and PCR amplification. The diagnostic performance of these methods was statistically compared. The results showed that *Acanthamoeba* infection was detected in 21 (19.1 %) of clinically suspected cases (110); 17 (81 %) of them were CLW and the remaining 4 (19 %) positive cases were NCLW. Regarding the used diagnostic methods, it was found that direct amplification of *Acanthamoeba* DNA bypassing nucleic acid extraction was superior to microscopy and culture in which 21 cases (19.1 %) were positive for *Acanthamoeba* by PCR compared to 19 positive cases by culture (17.3 %) and one case (0.9 %) by direct smear. The difference in detection rates between culture and direct smear was highly statistically significant (P=0.001). On the other hand, there was no significant difference in detection rates between culture and PCR (P=0.86). On using culture as the gold standard, PCR showed three false-positive samples that were negative by culture and one false-negative sample that was positive by culture. At the same time, direct smear showed 18 false-negative samples. The sensitivity, specificity, positive predictive value, negative predictive value, and diagnostic accuracy of PCR were 94.7, 96.7, 85.7, 98.9, and 96.4, respectively, while those of direct smear were 5.3, 100, 100, 83.5, and 83.6, respectively. In conclusion, direct amplification of *Acanthamoeba*-DNA bypassing DNA extraction is a reliable, specific, sensitive method in the diagnosis of *Acanthamoeba* keratitis in clinically suspected cases. It should set up in ophthalmological centers as an easy diagnostic tool.

Keywords *Acanthamoeba* keratitis · Diagnosis · Direct examination · Culture · PCR

N. M. El-Sayed (✉)
Parasitology Department, Research Institute of Ophthalmology, Giza, Egypt
e-mail: nag.elsaka@yahoo.com

N. M. El-Sayed
e-mail: nagelsaka@hotmail.com

M. S. Younis · A. M. Elhamshary · A. I. Abd-Elmaboud · S. M. Kishik
Parasitology Department, Faculty of Medicine- Benha University, Benha, Egypt

M. S. Younis
e-mail: msaadyounis@yahoo.com

A. M. Elhamshary
e-mail: azzaelhamshary@yahoo.com

A. I. Abd-Elmaboud
e-mail: draminaibrahim@yahoo.com

S. M. Kishik
e-mail: dr.shereen_kishik@yahoo.com

Introduction

Acanthamoeba keratitis (AK) is a painful sight-threatening ocular infection caused by free-living *Acanthamoeba* species (El-Sayed et al. 2012) which are abundant in the soil, dust, air, natural and treated water, seawater, domestic tap water, hospitals and dialysis units, eyewash stations, and contact lens cases (Johnston et al. 2009; Marciano-Cabral and Cabral 2003). Various species have been implicated in human infections: *Acanthamoeba astronyxis*, *Acanthamoeba castellanii*, *Acanthamoeba culbertsoni*, *Acanthamoeba polyphaga*, *Acanthamoeba hatchetti*, *Acanthamoeba rhysodes*, *Acanthamoeba lugdunensis*, *Acanthamoeba palestinensis*,

Acanthamoeba griffini, and *Acanthamoeba quina* (Khan 2006).

Acanthamoebae have two stages in their life cycle: a vegetative or trophozoite stage that reproduces by binary fission and feeds voraciously on bacteria and detritus present in the environment and a non-dividing cyst stage with a double-cyst wall, providing it with a high resistance to unfavoured and adverse environmental conditions, desiccation, and disinfecting compounds (Scheid and Schwarzenberger 2012).

AK was first recognized in the mid 1970s. Then, a dramatic increase in cases was associated with the increasing use of soft contact lens (in up to 93 % of cases); this is caused by improper lens handling and poor hygiene and also in the case of ocular trauma (Zhang et al. 2004). The disease is characterized by corneal inflammation, severe ocular pain, photophobia, central or paracentral stromal ring infiltrate, and recurrent breakdown of the corneal epithelium. The lesion, which is typically monolateral, is refractory to commonly used antibiotics and can provoke perforation and eventual vision loss (Marciano-Cabral and Cabral 2003; Visvesvara et al. 2007).

Laboratory diagnosis of AK relies on the demonstration of trophozoite or cyst in corneal scrapings under microscopic observation directly or isolated from the culture. Despite of the high specificity of culture-based methods, these assays need a long incubation time (14 days on average). Direct smears also can lead to misdiagnosis of *Acanthamoeba* in 60–70 % of AK cases (Sharma et al. 2004; Yera et al. 2007).

Long waiting time for the results obtained by cultivation may lead to a delay in proper treatment and, ultimately, worsening of the disease. Moreover, obtaining a sufficient volume of clinical specimen to facilitate direct smear or culture yields is challenging (Hammersmith 2006) and only few patients tolerate corneal scraping well. Thus, diagnostic methods that detect very few organisms in a clinical specimen are clearly advantageous. Therefore, polymerase chain reaction (PCR) procedures amplifying *Acanthamoeba* DNA have been developed to improve AK diagnosis and management (Yera et al. 2007; Thompson et al. 2008; Riviére et al. 2006; Goldschmidt et al. 2009; Qvarnstrom et al. 2006). Molecular methods offer the additional or alternative methods to microscopy and culture which have high sensitivity and specificity. These methods can be performed without a high experience in recognizing morphological features of *Acanthamoeba*. Moreover, they are very sensitive and permit the detection of fewer organisms per volume of sample analyzed than the morphological methods and they can also detect those cells that fail to grow at given culture conditions (Laummaunwai et al. 2012). So, this study evaluated the performance of direct amplification of *Acanthamoeba*-DNA bypassing DNA extraction in the diagnosis of AK in clinically suspected cases in comparison to traditional methods, direct microscopic examination, and in vitro culture.

Materials and methods

Subjects

The study was carried out on 110 patients attending the ophthalmic outpatient clinic of Benha University Hospital in the period from June 2011 to June 2012. The patients were clinically suspected to have AK, e.g., suffering from corneal inflammation with severe ocular pain and photophobia, kerato-conjunctivitis, resistant corneal ulcers or corneal abscesses, and not responding to antibacterial or antiviral treatment. Sixty-three of them (55 females and 8 males, and their age ranged from 14 to 40 years) were contact lens wearers, and the remaining 47 cases (21 females and 26 males, and their age ranged from 2 to 58 years) were non-contact lens wearers (NCLW). An informed consent was taken from all patients after explanation of the technique and the potential benefit of parasitological diagnosis. The study was approved by the Research Ethics Committee, Faculty of Medicine, Benha University, Egypt.

Using Kimura spatula, corneal scrapings were taken from the base and edge of the ulcers under aseptic condition, after installing local anesthetic solution (4 % Xylocaine) in the eye. Each scraped material was subjected to direct microscopic examination, cultivation onto the non-nutrient agar plate surface seeded with *Escherichia coli* (NNA–*E. coli*), and PCR amplification. For PCR analysis, corneal scraping was immersed in 200 μl of sterile phosphate-buffered saline (PBS) and stored at −20 °C until used. Specimen collections occurred prior to topical treatment to avoid possible false-negative results.

Direct microscopic examination

Corneal scraping was suspended into vials containing Page's saline and mixed well. With a sterile pipette, two drops of each specimen were placed onto a glass slide, covered by cover slips, and examined by light microscope using×40 magnification. Identification of *Acanthamoeba* was based on the characteristic patterns of locomotion, morphological features of the trophozoite and cyst forms.

Cultivation of corneal scraping (Init et al. 2010)

The obtained corneal sample was inoculated directly onto the NNA–*E. coli* plate surface and incubated in the incubator at 30 °C for 7 days. The plates were examined daily by the inverted microscope for *Acanthamoeba* growth. A drop of the fluid overlying the agar surface was placed on a glass slide, covered with a cover slip, and examined by light microscope using×40 magnification for the diagnostic criteria of *Acanthamoeba* organisms.

Preparation of positive control was, according to Thompson et al. (2008), with some modifications

A. castellanii was isolated and maintained by frequent subcultures every week on NNA–*E.coli* at 30 °C, and then *Acanthamoeba* growth was adapted to axenic cultivation on peptone, yeast extract, and glucose (PYG) liquid medium in a small screw-capped 10-ml tubes. The tubes were incubated at 37 °C at an angle of 5°, to allow amoebae to grow on the surface. After 24 to 48 h, luxuriant growth was obtained. Tubes were immersed in ice for 20–30 min to dislodge trophozoites from the side walls of the tubes, after which pellet was removed by centrifugation at 2,000*g* at 4 °C for 10–15 min. The pellets were washed three times in phosphate-buffered saline (PBS) (pH 7.2) and repelleted by repeated centrifugation, then resuspended in sterile PBS. The concentrations were adjusted to 1×10^4 trophozoites/ml sterile PBS by using a hemocytometer and stored at −20 °C until used for PCR.

Direct amplification of *Acanthamoeba*-DNA without nucleic acid extraction

Acanthamoeba DNA was amplified from corneal samples using KAPA Blood PCR Kit (Kapa Biosystems, Inc. USA) which has been validated for direct amplification from crude sample types, including buccal swabs and amniotic fluid. *Acanthamoeba* genus-specific primers used in this study were 18S ribosomal RNA (rRNA) gene primers (DF3 region) (forward primer 5′GGCCCAGATCGTTTACCGTGAA-3′ and reverse primer 5′TCTCACAAGCTGCTAGGGGAGTCA-3′) that were provided by Operon, Inc Huntsville, AL, USA.

Specimens for PCR processing were prepared by heating 0.3 ml of each corneal sample for 10 min at 98 °C and placing them on ice (heating helps to lyse cells to expose DNA). PCR reaction was set up by mixing 5 μl of each corneal sample, 25 μl of KAPA PCR Mix B (2X) (containing all PCR components, except the primers and template), and 2.50 μl of each primer. Then, PCR grade water was added to each tube-adjusted volume to 50 μl. Once pipetting was completed, the tubes were vortex before placing in the thermocycler. Amplification was done in rapid cycler PCR (G-Storm Thermal cycler, England). According to the manufacturer's instructions, the applied amplification program was 95 °C for 5 min as initial denaturation then cycling for 40 cycles. Each cycle consisted of 30 s of denaturation at 95 °C, 30 s of annealing at 52 °C, and 1 min of primer extension at 72 °C with an additional extension at 72 °C for 10 min after the last cycle. Positive and negative controls were used for each run. Positive control was 1×10^4 trophozoites/ml sterile PBS while the negative control was the PCR mixture without corneal sample and with nuclease free water instead to detect the possible contaminations.

Post-PCR processing and analysis of the amplified products

After completion of the PCR program, the obtained amplified products were centrifuged at 14,000*g* speed to obtain the most compact pellet of organic debris and facilitate recovery of the amplicon containing supernatant. The amplified DNA was analyzed by electrophoresis. Ten microliter of each reaction mixture and 1,000-bp ladder (molecular weight marker) was separated on 2 % agarose gel contain 0.3 μg/ml of ethidium bromide. The bands were visualized using UV Transeilluminator (254 nm) and photographed and analyzed using digital camera 8 megapixel. The photo was transferred to computerized analysis using Gel Documentation System (Alpha Inotech). The positive control lane showed a specific band at 500 bp, the negative control lane was free from any band, and samples showing a band opposite to the positive control band were considered as positive (Fig. 1).

Statistical analysis

It was conducted using chi-square (χ^2) by SPSS V17. Values were considered statistically significant when the probability value was less than 0.05 ($P<0.05$) and highly significant when the P value was 0.001. For the diagnostic tests, the variables measured were the numbers of true positives (TP), true negatives (TN), false positives (FP), and false negatives (FN). Sensitivity was then calculated as TP / (TP+FN)×100, specificity as TN / (TN+FP)×100, positive predictive value (PPV) as TP / (TP+FP)×100, the negative predictive value (NPV) as TN / (FN+TN)×100, and diagnostic accuracy (DA) as TP+TN / total no. of patients×100.

Results

Results are shown in Tables 1, 2, and 3 and Fig. 1.

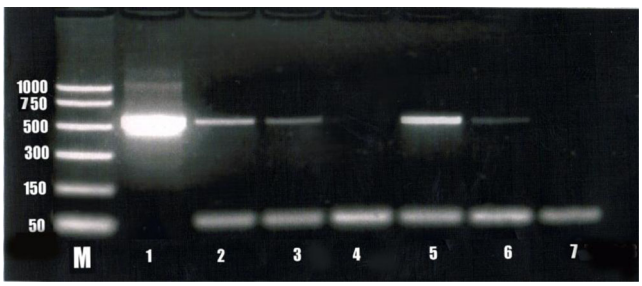


Fig. 1 Agarose gel electrophoresis of the amplified products of *Acanthamoeba*-DNA in corneal scraping samples. *M* molecular size marker 1,000 bp. *Lane 1*: positive control (500 bp band), *Lanes 2*, *3*, *and 6*: positive cases with low load of *Acanthamoeba* in the samples. *Lane 5*: positive case with high load of *Acanthamoeba* in the samples. *Lane 4*: negative case. *Lane 7*: negative control

Table 1 Percentage of *Acanthamoeba* infection among contact lens wearers (CLW) and non-contact lens wearers (NCLW) obtained by the combination of three diagnostic tests used in this study

Acanthamoeba	Keratitic patients				Total		$\chi 2$	*P* value
	NCLW		CLW					
	No.	%	No.	%	No.	%		
Positive	4	19	17	81	21	19.1	5.947	<0.05[a]
Negative	43	48.3	46	51.7	89	80.9		
Total	47	42.7	63	57.3	110	100		

[a] Significant difference

Discussion

The most important step in the diagnosis of AK is to suspect it, especially in contact lens wearers (CLW) with the corneal involvement, presenting with the pain disproportional to the clinical findings. As evidence of the importance of collaboration between clinicians and laboratory technicians, the correct diagnosis of amoebic infection was achieved using parasitological methods that allowed direct microscopic identification of the pathogens and in vitro culture. In addition, integration of a PCR method with the conventional parasitological techniques may complete the diagnostic protocol in the case of AK and offer information on the pathogenicity of the amoebic isolates (Qvarnstrom et al. 2006; Gatti et al. 2010). Each of these diagnostic methods has strengths and weaknesses in terms of test parameters, cost, and technical complexity.

The results of the present study showed that *Acanthamoeba* infection was detected in 21 (19.1 %) of clinically suspected patients (110); 17 (81 %) of them were CLW and the remaining 4 (19 %) positive cases were NCLW. These results revealed a significant association between AK and wearing of contact lenses ($P<0.05$) which appears to be an important risk factor in *Acanthamoeba* infection. This significant association was confirmed previously by other investigators who found that wearing of contact lens was associated with 62.5 to 95 % of AK cases (Gupta and Aher 2009; Ibrahim et al. 2009; Wanachiwanawin et al. 2012).

It is widely believed that manipulation of the contact lenses may result in epithelial breaks that transmit infectious *Acanthamoeba* trophozoites to the eye (Ibrahim et al. 2009). Additionally, contact lenses cause chronic hypoxic stress on the corneal epithelium which leads to decreased corneal sensitivity, decreased epithelial mitosis and adhesion, premature desquamation of epithelial cells, increased epithelial fragility, epithelial microcystic edema, and significant thinning of the epithelial cell layer (Liesegang 2002; Myrowitz et al. 2002). In fact, contact lenses can stimulate the expression of glycoproteins on the corneal epithelium. This in turn might exacerbate the infectious process, as mannosylated proteins promote the binding of *Acanthamoeba* trophozoites to the corneal epithelium via a mannose-binding protein (mannose receptor) that is expressed on the *Acanthamoeba* cell membrane (Clarke and Niederkorn 2006).

Regarding the diagnostic methods used in this study for the detection of *Acanthamoeba* in corneal scrapings of clinically suspected patients (110), it was found that direct amplification of *Acanthamoeba* DNA bypassing nucleic acid extraction was superior to microscopy and culture in which 21 cases (19.1 %) were positive for *Acanthamoeba* by PCR compared to 19 positive cases by culture (17.3 %) and one case (0.9 %) by direct smear. The difference in detection rates between culture and direct smear was highly statistically significant ($P=0.001$). On the other hand, there was no significant difference in detection rates between culture and PCR ($P=0.86$). The variability of detection rates may be attributed to variable sensitivity of different diagnostic methods and technical expertise of the personnel performing the diagnosis.

In the current study, direct amplification of *Acanthamoeba* DNA bypassing nucleic acid extraction using a commercial KAPA PCR kit proved to be a simple and efficient method for the detection of *Acanthamoeba* even a single cyst did not require high-cost reagents or complicated procedures to

Table 2 Results of direct smear and PCR in detection of *Acanthamoeba* among keratitic patients in comparison to culture

Applied tests		Culture			*P* value
		Positive %	Negative %	Total %	
Direct smear	Positive (%)	1 (0.9 %)	0 (0.0 %)	1 (0.9 %)	0.001[a]
	Negative (%)	18 (16.4 %)	91 (82.7 %)	109 (99.1 %)	
	Total (%)	19 (17.3 %)	91 (82.7 %)	110 (100 %)	
PCR	Positive (%)	18 (16.4 %)	3 (2.7 %)	21 (19.1 %)	0.86[b]
	Negative (%)	1 (0.9 %)	88 (80.0 %)	89 (80.9 %)	
	Total (%)	19 (17.3 %)	91 (82.7 %)	110 (100 %)	

[a] Highly significant difference

[b] No significant difference

Table 3 Sensitivity, specificity, predictive values, and diagnostic accuracy of direct smear and PCR in relation to culture for *Acanthamoeba* detection

Technique	Sensitivity	Specificity	PPV	NPV	Accuracy
Direct smear (%)	5.3	100	100	83.5	83.6
PCR (%)	94.7	96.7	85.7	98.9	96.4

extract DNA and offered a much more rapid time. The availability of PCR results within several hours of sample taking allows the clinicians to adapt their treatment very rapidly with a potential positive effect on the final prognosis. These results were in accordance with those of Zhao et al. (2014) who found that the direct PCR assay without template DNA extraction using specific primers for fungi, bacteria, *herpes simplex* virus-1, and *Acanthamoeba* is a rapid diagnostic technique with high sensitivity and specificity. The specificity of 18S rRNA gene primers used in this study was validated previously by Pasricha et al. (2003), Sharma et al. (2004), and Niyyati et al. (2009) who confirmed that PCR by using these primers could provide a high sensitivity and specificity for the detection of *Acanthamoeba* in corneal scrapes in a short period. These primers are also able to detect a very small amount of *Acanthamoeba* DNA (1–2 pg) (Yera et al. 2007).

Several investigators have suggested the most accurate technique for the diagnosis of acanthamoebiasis still requires in vitro cultivation (Niyyati et al. 2009; Marciano-Cabral and Cabral 2003) due to its low cost and simplicity. Moreover, in vitro culture amplified the parasite number, thus making microscopic detection less time consuming. Other benefits include the possibility of maintaining and expanding isolates in culture for further studies. So, in this study, culture was used as a gold standard to evaluate the sensitivities of other detection methods.

Based on the result of culture, direct smear showed 18 false-negative samples that were positive by culture, yielding a very low sensitivity (5.3 %) in detection of *Acanthamoeba* organisms. This finding is in agreement with Sharma et al. (2000) and Boggild et al. (2009) who found that direct smear had the poorest diagnostic sensitivity (33–55 %) compared to culture and PCR. Moreover, Yera et al. (2008) revealed that all of corneal scrapes of AK patients were negative by direct examination. The difficulty in *Acanthamoeba* detection by direct examination may be attributed to small corneal sample needed for direct smear which may contain a few numbers of *Acanthamoebae* and inability of laboratory technicians to recognize these pathogens morphologically in biological samples (Marciano-Cabral and Cabral 2003; Qvarnstrom et al. 2006). In spite of direct microscopic examination of a corneal smear can provide results in a short span of time, enabling the clinician to start empirical treatment, its low sensitivity highlighted the danger of relying on it for diagnosis of AK infection.

Although culture is considered a reliable method of diagnosis, it requires a special medium and the results usually need a long incubation time (few days for trophozoites and 1 to 2 weeks for encystations) and frequent microscopic observations (Lek-Uthai et al. 2009). Furthermore, it may produce false-negative results as observed in this study in which culture failed to detect three positive specimens by PCR. The culture failure might be due to the difficulty of obtaining sufficient corneal scrapings (Xuejun et al. 2009), low parasitic load, and the use of antiseptic or antibiotics prior to sampling (Petry et al. 2006) and also due to weakness of the *Acanthamoeba* strains isolated or resistance of the corneal epithelium to invasion of the organism (Mathers et al. 2000). In addition, cyst forms which do not excyst and trophozoites, which are damaged during sample preparation, might not be detected through culture (Riviére et al. 2006).

Comparing to in vitro culture, PCR showed three false-positive samples that were negative by culture and one false-negative sample that were positive by culture yielding the best diagnostic results for sensitivity (94.7 %), specificity (96.7 %), positive predictive value (85.7 %), negative predictive value (98.9 %), and diagnostic accuracy (96.4 %). The obtained false-negative results may be due to insufficient DNA material on the corneal scraping and the resistance of the cystic form to expose DNA (Goldschmidt et al. 2008) or due to nuclear material degradation in aging specimens. Other factors, such as local anesthetics used for therapeutic treatment of AK, are also known to act as PCR inhibitors. Therefore, corneal specimens should be collected after rinsing the eye surface properly (Goldschmidt et al. 2006; Seitzman et al. 2006). On the other hand, the obtained false-positive results may be due to the possibility of contamination from the carryover of product DNA via pipettes or even viral shedding by the technician.

In spite of PCR procedure has proved its efficiency in diagnosing AK, with a real benefit for patient care, the use of this technique is limited to research labs, as it involves a high cost as well as trained personnel. Disadvantages of PCR can be traced to its high sensitivity and specificity. Because PCR can potentially detect the presence of a single genome, laboratory contamination is a substantial hazard and can undermine the usefulness of PCR. The high specificity of PCR can also be problematic in which a different primer set must be used for each organism in the differential diagnosis. Unlike culture techniques, which can detect a wide range of organisms, each organism must be assayed individually in PCR (Lohmann et al. 2000).

In conclusion, direct amplification of *Acanthamoeba* DNA bypassing DNA extraction using the commercial KAPA PCR kit is a reliable, specific, sensitive method in the diagnosis of AK in clinically suspected cases and does not require high-cost reagents or complicated procedures to extract DNA. It should set up in ophthalmological centers as an easy diagnostic tool.

Acknowledgments The authors are grateful to Assistant Prof. Naglaa Fathy Alhusseini, Department of Biochemistry, Faculty of Medicine, Benha University, Egypt, for her technical advice.

References

Boggild AK, Martin DS, Lee TYL, Yu B, Low DE (2009) Laboratory diagnosis of amoebic keratitis: comparison of four diagnostic methods for different types of clinical specimens. J Clin Microbiol 47(5):1314–1318

Clarke DW, Niederkorn JY (2006) The pathophysiology of *Acanthamoeba* keratitis. Trends Parasitol 22(4):175–180

El-Sayed NM, Ismail KA, Ahmed SAG, Hetta MH (2012) In vitro amoebicidal activity of ethanol extracts of *Arachis hypogaea L., Curcuma longa L.* and *Pancratium maritimum L.* on *Acanthamoeba castellanii* cysts. Parasitol Res 110(5):1985–1992

Gatti S, Rama P, Matuska S, Berrilli F, Cavallero A, Carletti S, Bruno A, Maserati R, Di Cave D (2010) Isolation and genotyping of *Acanthamoeba* strains from corneal infections in Italy. J Med Microbiol 59:1324–1330

Goldschmidt P, Rostane H, Saint-Jean C, Batellier L, Alouch C, Zito E, Bourcier T, Laroche L, Chaumeil C (2006) Effects of topical anesthetics and fluorescein on the real-time PCR used for the diagnosis of Herpes viruses and *Acanthamoeba* keratitis. Br J Ophthalmol 90(11):1354–1356

Goldschmidt P, Degorge S, Saint-Jean C, Year H, Zekhnini F, Batellier L, Laroche L, Chaumeil C (2008) Resistance of *Acanthamoeba* to classic DNA extraction methods used for the diagnosis of corneal infections. Br J Ophtalmol 92(1):112–115

Goldschmidt P, Degorge S, Benallaoua D, Saint-Jean C, Batellier L, Alouch C, Laroche L, Chaumeil C (2009) New tool for the simultaneous detection of 10 different genotypes of *Acanthamoeba* available from the American type culture collection. Br J Ophthalmol 93:1096–1100

Gupta S, Aher A (2009) *Acanthamoeba* keratitis—a case report. People's J Sci Res 2(2):9–11

Hammersmith KM (2006) iagnosis and management of *Acanthamoeba* keratitis. Curr Opin Ophthalmol 17(4):327–331

Ibrahim YW, Boase DL, Cree IA (2009) How could contact lens wearers be at risk of *Acanthamoeba* infection? A review. J Optom 2(2):60–66

Init I, Lau YL, Arin-Fadzlun A, Foead AI, Neilson RS, Nissapatorn V (2010) Detection of free living *Amoebae*, *Acanthamoeba* and *Naegleria*, in swimming pools, Malaysia. Trop Biomed 27(3):566–577

Johnston SP, Sriram R, Qvarnstrom Y, Roy S, Verani J, Yoder J, Lorick S, Roberts J, Beach MJ, Visvesvara G (2009) Resistance of *Acanthamoeba* cysts to disinfection in multiple contact lens solutions. J Clin Microbiol 47(7):2040–2045

Khan NA (2006) *Acanthamoeba*: biology and increasing importance in human health. FEMS Microbiol Rev 30:564–595

Laummaunwai P, Ruangjirachuporn W, Boonmars T (2012) A simple PCR condition for detection of a single cyst of *Acanthamoeba* species. Parasitol Res 110(4):1569–1572

Lek-Uthai U, Passara R, Roongruangchai K (2009) Morphological features of *Acanthamoeba* causing keratitis contaminated from contact lens cases. J Med Assoc Thai 92(7):156–163

Liesegang TJ (2002) Physiologic changes of the cornea with contact lens wear. CLAO J 28:12–27

Lohmann CP, Linde HJ, Reischl U (2000) Improved detection of microorganisms by polymerase chain reaction in delayed endophthalmitis after cataract surgery. J Ophthalmol 107(6):1047–1051

Marciano-Cabral F, Cabral G (2003) *Acanthamoeba* spp as agents of disease in humans. Clin Microbiol Rev 16(2):273–307

Mathers WD, Nelson SE, Lane JL, Wilson ME, Allen RC, Folberg R (2000) Confirmation of confocal microscopy diagnosis of *Acanthamoeba* keratitis using polymerase chain reaction analysis. Arch Ophthalmol 118(2):178–183

Myrowitz EH, Melia M, O'Brien TP (2002) The relationship between long term contact lens wear and corneal thickness. CLAO J 28:217–220

Niyyati M, Lorenzo-Morales J, Mohebali M, Rezaie S, Rahimi F, Babaei Z, Martín-Navarro CM, Farnia S, Valladares B, Rezaeian M (2009) Comparison of a PCR-based method with culture and direct examination for diagnosis of *Acanthamoeba* keratitis. Iranian J Parasitol 4(2):38–43

Pasricha G, Sharma S, Garg P, Aggarwal RK (2003) Use of 18S rRNA gene-based PCR assay for diagnosis of *Acanthamoeba* keratitis in non-contact lens wearers in India. J Clin Microbiol 41:3206–3211

Petry F, Torzewski M, Bohl J, Wilhelm-Schwenkmezger T, Scheid P, Walochnik J, Michel R, Zöller L, Werhahn KJ, Bhakdi S, Lackner KJ (2006) Early diagnosis of *Acanthamoeba* infection during routine cytological examination of cerebrospinal fluid. J Clin Microbiol 44(5):1903–1904

Qvarnstrom Y, Visvesvara GS, Sriram R, da Silva AJ (2006) Multiplex real-time PCR assay for simultaneous detection of *Acanthamoeba* spp., *Balamuthia mandrillaris*, and *Naegleria fowleri*. J Clin Microbiol 44:3589–3595

Riviére D, Szczebara F, Berjeaud J, H'echard Y (2006) Development of real-time PCR assay for quantification of *Acanthamoeba* trophozoites and cysts. J Microbiol Meth 64:78–83

Scheid P, Schwarzenberger R (2012) *Acanthamoeba* spp. as vehicle and reservoir of adenoviruses. Parasitol Res 111(1):479–485

Seitzman GD, Cevallos V, Margolis TP (2006) Rose bengal and lissamine green inhibit detection of herpes simplex virus by PCR. Am J Ophthalmol 141:756–758

Sharma S, Garg P, Rao GN (2000) Patient characteristics, diagnosis, and treatment of non-contact lens related Acanthamoeba keratitis. Br J Ophthalmol 84(10):1103–1108

Sharma S, Pasricha G, Das D, Aggarwal RK (2004) *Acanthamoeba* keratitis in non-contact lens wearers in India: DNA typing-based validation and a simple detection assay. Arch Ophthalmol 122(10):1430–1434

Thompson PP, Kowalski RP, Shanks RM, Gordon YJ (2008) Validation of real-time PCR for laboratory diagnosis of *Acanthamoeba* keratitis. J Clin Microbiol 46:3232–3236

Visvesvara GS, Moura H, Schuster FL (2007) Pathogenic and opportunistic free-living amoebae: *Acanthamoeba* spp., *Balamuthia mandrillaris*, *Naegleria fowleri*, and *Sappinia diploidea*. FEMS Immunol Med Microbiol 50:1–26

Wanachiwanawin D, Booranapong W, Kosrirukvongs P (2012) Clinical features of *Acanthamoeba* keratitis in contact lens wearers and non wearers. Southeast Asian J Trop Med Pub Health 43(3):549–456

Xuejun Z, Xiaoyan L, Xiaoji S, Guoxing X, Jianzhang H (2009) Application of 18S rDNA PCR technique in the laboratory diagnosis of *Acanthamoeba* keratitis in the clinical. Guoji Yanke Zazhi 9:715–718

Yera H, Zamfir O, Bourcier T, Ancelle T, Batellier L, Dupouy-Camet J, Chaumeil C (2007) Comparison of PCR, microscopic examination and culture for the early diagnosis and characterization of *Acanthamoeba* isolates from ocular infections. Eur J Clin Microbiol Infect Dis 26(3):221–224

Yera H, Zamfir O, Bourcier T, Viscogliosi E, Noel C, Jean D, Chaumeil C (2008) The genotypic characterization of *Acanthamoeba* isolates from human ocular samples. Br J Ophthalmol 92(8):1139–1141

Zhang Y, Sun X, Wang Z, Li R, Luo S, Jin X, Deng S, Chen W (2004) Identification of 18S ribosomal DNA genotype of *Acanthamoeba* from patients with keratitis in North China. Invest Ophthalmol Vis Sci 45(6):1904–1907

Zhao G, Zhai H, Yuan Q, Sun S, Liu T, Xie L (2014) Rapid and sensitive diagnosis of fungal keratitis using direct PCR without template DNA extraction. Clin Microbiol Infect. doi:10.1111/1469-0691.12571