

Molecular diagnosis of *Acanthamoeba* keratitis: evaluation in rat model and application in suspected human cases

Adriana Oliveira Costa¹ · Cinthia Furst² · Lucas Oliveira Rocha³ ·
Cecília Cirelli¹ · Carolina Neris Cardoso¹ · Fagner Salmazo Neiva³ ·
Cynara Oliveira Possamai² · Daniel de Assis Santos⁴ · Vanete Thomaz-Soccol³

Received: 7 October 2016 / Accepted: 13 February 2017 / Published online: 1 March 2017
© Springer-Verlag Berlin Heidelberg 2017

Abstract *Acanthamoeba* keratitis (AK) is a progressive corneal infection that demands rapid and sensitive techniques for diagnosis to avoid risk of visual impairment. We evaluated two DNA extraction techniques and a semi-nested-PCR (snPCR) targeting the 18S rRNA gene to detect *Acanthamoeba* cysts and trophozoites. The most effective protocol was evaluated in samples of corneal scrapings and biopsies from an AK rat model and applied to diagnosis of human cases of AK. DNA extraction performed with a commercial kit based on DNA binding to magnetic beads was more efficient than a method based on alkaline lysis, allowing the detection of one trophozoite and one cyst of *Acanthamoeba* in samples prepared from cultures. This technique and sn-PCR were applied in corneal scrapings of rats experimentally infected with *Acanthamoeba* ($n = 6$), resulting in 100% of positivity, against 16.7% ($n = 6$) of positive identification in culture method using non-nutrient agar (NNA) with *Escherichia coli*. Corneal biopsies from rats were also tested ($n = 6$) and resulted in positivity in all samples in both molecular and culture methods. Eight out of ten presumptive

human cases of *Acanthamoeba* keratitis were also confirmed by sn-PCR of scraping samples, while the culture method was positive in only four cases. We discuss that animal model of AK can be an efficient tool to validate diagnostic methods and conclude that DNA extraction with the kit and snPCR protocol described here is an effective alternative for diagnosis of AK.

Keywords *Acanthamoeba* keratitis · Rat model · Diagnosis · Semi-nested PCR · Culture

Introduction

Acanthamoeba keratitis (AK) is a progressive corneal infection that can cause irreversible vision impairment. The illness is characterized by severe pain, usually disproportionate to the clinical signs, punctate keratopathy, tearing, photophobia, and stromal ring infiltrates (Maycock et al. 2016). Contact lenses users are more likely to acquire AK, representing 62 to 71% of cases (Radford et al. 2002). Corneal traumatic lesions caused by a foreign body followed by exposure to contaminated water can also be a source of AK (Marciano-Cabral and Cabral 2003).

AK requires early diagnosis for a rapid therapeutic intervention, since the prognosis worsens if an effective therapy is not introduced within 3 weeks (Claerhout et al. 2004). History of contact lens use, ocular trauma, or unsuccessful treatments for other disorders such as herpes simplex virus and fungal infections should suggest the possibility of AK (Bacon et al. 1993; Sharma et al. 2000; Sun et al. 2006). Thus, laboratory diagnosis is necessary to confirm the infection. Culture of corneal scrapings is the gold standard for definitive diagnosis of AK (Schuster and Visvesvara 2004) and is also simple and of low cost. However, culture results can take 1 to 10 days,

✉ Adriana Oliveira Costa
adrincosta@gmail.com

¹ Departamento de Análises Clínicas e Toxicológicas, Faculdade de Farmácia, Universidade Federal de Minas Gerais, Av. Presidente Antônio Carlos, 6627, Belo Horizonte, Minas Gerais 31270-901, Brazil

² Departamento de Patologia, Universidade Federal do Espírito Santo, Vitória, Espírito Santo, Brazil

³ Departamento de Patologia Básica, Setor de Ciências Biológicas, Universidade Federal do Paraná, Curitiba, Paraná, Brazil

⁴ Departamento de Microbiologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil

delaying initiation of adequate treatment (Khairnar et al. 2011). Besides, its sensitivity is not high, being reported to range from 7% (Yera et al. 2007) to 57% (Dart et al. 2009).

Polymerase chain reaction-based methods can provide a rapid and sensitive mean of detecting *Acanthamoeba* in corneal scrapings compared to microbiological and culture methods (Schroeder et al. 2001; Yera et al. 2007; Dhivya et al. 2007; Khairnar et al. 2011). Studies have reported limits of detection of five trophozoites (Khan 2001) and one trophozoite (Schroeder et al. 2001) for PCR using regions of the 18S-rRNA gene as a target. Semi-nested PCR (snPCR) developed by Dhivya et al. (2007) has shown higher sensitivity than uniplex PCR. However, strategies for efficient DNA extraction are needed to avoid false negative results, especially for encysted forms of *Acanthamoeba* also found in corneal tissue, which are particularly resistant to DNA-extraction procedures validated for other microorganisms (Goldschmidt et al. 2008).

This study aimed to evaluate a protocol for molecular detection of *Acanthamoeba* in samples of cornea for the diagnosis of AK. Initially, two DNA extraction techniques along with snPCR described by Dhivya et al. (2007) were tested for detection of trophozoites and cysts from cultures. The most effective protocol was evaluated in corneal samples from experimentally infected rats and subsequently applied to diagnose presumptive cases of AK.

Materials and methods

Sample preparation

Trophozoites of an *Acanthamoeba polyphaga* strain (ATCC 30461) cultured at 30 °C in proteose peptone-yeast extract-glucose (PYG) medium (Visvesvara and Balamuth 1975) were used in the experiments. Cysts were obtained after incubating trophozoites in Neff saline (Neff et al. 1964) for 72 h at 30 °C.

Trophozoites and cysts were quantified in a Neubauer counting chamber, and microtubes containing 50 µL of ultrapure water were inoculated with 1, 5, 10, or 100 cells, in triplicate. Lower quantities (1, 5, and 10) were prepared by microscopic examination of drops of 3–5 µL of diluted suspension on a microscope slide. After identifying the desired quantities, the drop was aspirated and transferred to the microtube. Drops without trophozoites or cysts were also transferred to microtubes to be used as negative controls.

DNA extraction methods

Two protocols of DNA extraction were tested: (1) alkaline lysis and precipitation with ammonium acetate, based on a technique used for DNA extraction of *Entamoeba histolytica*

cysts in feces (Vianna et al. 2009), and (2) a method using the ChargeSwitch® gDNA Mini Tissue Kit (Invitrogen), which is based on DNA binding to magnetic beads.

In the alkaline lysis method, 200 µL of lysis buffer (50 mM glucose, 25 mM Tris HCl pH 8, 10 mM EDTA) and 400 µL of 0.2 mM NaOH with 1% SDS were added to the sample and subjected to seven freeze/thaw cycles (5 min in liquid nitrogen and 5 min at 99 °C). After addition of 300 µL of 7.5 mM ammonium acetate, samples were homogenized and centrifuged at 10,000×g for 5 min. About 700 µL of the supernatant was transferred to a microtube, and 500 µL of isopropanol was added. The mixture was gently homogenized, incubated at –20 °C for 15 min, and centrifuged at 10,000×g for 15 min. The supernatant was discarded and the pellet washed in 70% ethanol. After drying the pellet at ambient temperature, DNA was re-suspended with 25 µL ultrapure water.

For the DNA extraction using the kit, samples were subjected to seven freeze/thaw cycles (5 min in liquid nitrogen and 2 min at 37 °C) in 500 µL of lysis buffer provided in the kit. The subsequent steps of extraction were performed as recommended by the manufacturer's protocol.

DNA samples from microorganisms known to cause corneal infection were also used to evaluate specificity (*Candida albicans*, *Fusarium solani*, *Aspergillus fumigatus*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, Herpes simplex virus type 1).

Amplification reactions

Briefly, the first round of PCR used the primers JDP1 and JDP2 (Schroeder et al. 2001) in reactions performed on a final volume of 15 µL with 4 mM MgCl₂, 0.2 mM dNTP, 0.6 mg/µL bovine serum albumin (BSA), 0.5 µM of each primer, 0.6 U of Platinum® Taq DNA Polymerase (Invitrogen), and 5 µL of DNA. The amplification profile was 94 °C for 7 min, 72 °C for 1 min, 25 cycles at 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 2 min followed by a final extension step of 72 °C for 10 min. The snPCR was performed as described by Dhivya et al. (2007) with minor modifications. Two microliters of PCR product was used for the snPCR carried out on 15 µL of reaction containing 1.6 mM MgCl₂, 0.2 mM dNTP, 0.5 µM of the inner forward primer A1 and the outer reverse primer JDP2, and 0.5 U of Platinum® Taq DNA Polymerase (Invitrogen). Amplification was performed at 95 °C for 5 min, followed by 20 cycles at 95, 64, and 72 °C for 45 s each and a final extension at 72 °C for 5 min. All amplification reactions were performed in a thermocycler Biocycler, MJ96 (Biosystems®). The products of snPCR were separated by electrophoresis in 1.5% agarose gel stained with ethidium bromide.

Experimental keratitis in rats

Six female Wistar rats approximately 60 days old were anesthetized with 60 mg/kg ketamine HCl (Vetanarcol®, König do Brasil, Brazil) and 7.5 mg/kg xylazine (Dopaser®, Calier, Spain) by intraperitoneal injection. The left eye was washed three times with sterilized saline solution and then anesthetized with topical 2% lidocaine. Intrastromal inoculation was performed with 2 µL of suspension containing 10⁵ trophozoites of the *Acanthamoeba polyphaga* ATCC 30461 in Page's saline (2.5 mM NaCl, 1 mM KH₂PO₄, 0.5 mM Na₂HPO₄, 40 mM CaCl₂, and 20 mM MgSO₄), using a microliter 30G needle, similar to the procedures of Ren and Wu (2010). Two animals were inoculated with Page's saline only as controls. Six days postinoculation, the lesions were evaluated and the rats euthanized with a double dose of anesthetic before performing corneal scrapings and biopsy. The lesions were graded as follows: Grade I, point of opacity limited to the region of inoculum, less than 1 mm in diameter; Grade II, point of opacity greater than 1 mm, clearly surpassing the initial point of inoculum, but not occupying the entire cornea; and Grade III, extensive damaged area, involving more than 50% of the cornea.

Detection of *Acanthamoeba* in rat corneal tissue

Each rat inoculated with *Acanthamoeba* was positioned under a stereomicroscope; the head was immobilized with tape, and the eye washed with sterilized saline. Using a #15 scalpel blade, the injured area of the cornea was scraped three times at paralleled sites at the lesion. Each corneal scraping was inoculated in (1) a tube with 2 mL of PYG medium, (2) a plate non-nutrient agar (NNA) with heated-inactivated *Escherichia coli*, and (3) a microtube containing 50 µL of Page's saline. The scalpel was flame-sterilized between each procedure. For biopsy, a 3-mm fragment of the cornea, incorporating both corneal infiltrate and adjacent clear cornea, was excised and removed with a fine tip tweezers to a sterilized lamina. The

fragment was separated into three parts for culture in PYG, culture in NNA, and inoculation in a microtube with Page's saline, as previously described for scrapings. Corneas inoculated only with Page's saline were submitted to the same procedures as controls. Cultures were incubated at 30 °C and examined from day 5 to day 20 following the seeding. Samples collected in the microtubes with 50 µL of Page's saline were stored at -20 °C until DNA extraction using ChargeSwitch® gDNA Mini Tissue Kit (Invitrogen) and snPCR.

Diagnosis of human cases of *Acanthamoeba* keratitis

Ten presumptive human cases of AK admitted in Ophthalmology Sector of Cassiano Antônio de Moraes University Hospital, Vitória, Espírito Santo, Brazil, during the period of March 2010 and May 2012, were evaluated by culture and PCR/snPCR. Corneal samples were seeded in soy-agar plates with *Escherichia coli*, as described by Costa et al. (2010). A second scraping was transferred to microtubes with 200 µL de Page's saline and immediately transported to the laboratory for centrifugation at 5000×g by 5 min. About 150 µL of the supernatant was discarded and the microtube was stocked at -20 °C until performing DNA extraction, which was carried out with ChargeSwitch® gDNA Mini Tissue Kit (Invitrogen).

Results

The snPCR has detected one trophozoite in two out of three repetitions using both DNA extraction methods (Table 1). When cysts were used for DNA extraction, positivity by snPCR was achieved with 100 forms treated by alkaline lysis extraction and 1 to 10 forms subjected to the commercial kit (although this did not occur in all repetitions) (Table 1). The samples made of ultrapure water (negative controls) and DNA samples obtained from fungi, bacteria, and virus (specificity controls) had a negative result in the tests.

Table 1 snPCR positivity for samples containing 1, 5, 10, and 100 *Acanthamoeba* trophozoites or cysts after DNA extraction by two different methods

Reaction	Forms	Alkaline lysis ^b				Extraction kit ^c			
		1	5	10	100	1	5	10	100
snPCR ^a	Trophozoites	++ -	++ -	+++	+++	++ -	++ -	+++	+++
	Cysts	---	---	---	+++	+ - -	++ -	++ -	+++

+ positive result, - negative result

^a snPCR targeting the ASA-S1 fragment of 18S rRNA (Dhivya et al. 2007) in triplicate samples

^b Extraction performed by freeze/thaw cycles in glucose-Tris-EDTA buffer containing NaOH and SDS, followed by precipitation in ammonium acetate

^c Extraction with the ChargeSwitch® gDNA Mini Tissue (Invitrogen) after freeze/thaw cycles

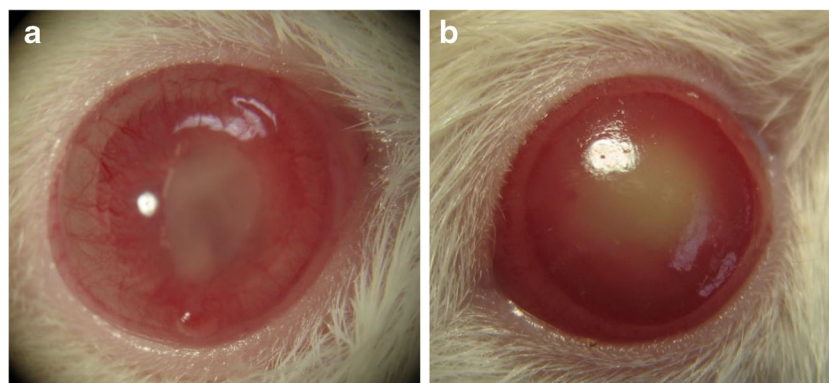


Fig. 1 *Acanthamoeba* keratitis induced in Wistar rats by intrastromal inoculation of 10^5 trophozoites of *A. polyphaga* strain ATCC 30461. Lesions were evaluated 5 days after the inoculation. Lesions classified

as grade II, with the point of opacity greater than 1 mm, clearly exceeding the initial point of inoculum (a) and grade III, showing an extensive damaged area and involvement of more than 50% of the cornea (b)

Keratitis induced in Wistar rats resulted in corneal lesions from grades I to III in all animals (Fig. 1, Table 2). Corneal inoculation with Page's saline resulted in no lesions. Table 2 outlines results of the cultures and snPCR, using corneal scraping or corneal biopsy from the animals. For corneal scraping samples, culture in PYG was negative, culture in NNA was positive in only one sample, and snPCR showed signs of amplification in all six samples. In corneal biopsy material, all six samples were positive in NNA cultures, while four samples were detected by culture in PYG. All corneal biopsy samples were positive for *Acanthamoeba* by snPCR. Samples from rat cornea inoculated with Page's saline were negative in all diagnostic methods.

Four out of ten corneal scrapings of presumptive cases of *Acanthamoeba* keratitis were positive in culture method and eight were positive in snPCR (Table 3).

Discussion

In recent years, both conventional PCR and real-time PCR have been reported to be efficient techniques to detect *Acanthamoeba* in corneal tissue (Thompson et al. 2008; Khairnar et al. 2011; Laummaunwai et al. 2012). Conventional PCR is widespread, and lower cost equipments and reagents have recently become available. However, limitations such as the low number of amoebae in the corneal scrapings and the greater resistance of cysts to extraction and release of DNA must be considered in establishing a protocol using molecular techniques. In the present study, we have evaluated a DNA extraction method that was efficient to detect one trophozoite and one cyst in snPCR. We have also validated it in corneal samples of animal and, subsequently, in human scrapings.

Table 2 Results of culture and snPCR for corneal scraping and corneal biopsy of Wistar rats with experimentally induced *Acanthamoeba* keratitis

Animal	Lesion grade ^a	Corneal scraping			Corneal biopsy		
		Culture NNA ^b	Culture PYG ^c	snPCR ^d	Culture NNA ^b	Culture PYG ^c	snPCR ^d
1	II	+	–	+	+	–	+
2	I	–	–	+	+	+	+
3	II	–	–	+	+	+	+
4	III	–	–	+	+	+	+
5	III	–	–	+	+	+	+
6	III	–	–	+	+	+	+
Rate	NA	1/6 (16.7%)	0/6 (0%)	6/6 (100%)	6/6 (100%)	5/6 (83.3%)	6/6 (100%)

NA not applicable, + positivity, – negativity

^a Lesion evaluated 5 days after inoculation, as follows: Grade I, point of opacity limited to the region of inoculum, less than 1 mm in diameter; Grade II, point of opacity greater than 1 mm, clearly surpassing the initial point of inoculum, but not occupying the entire cornea; Grade III, extensive damaged area, involvement of more than 75% of the cornea

^b Culture in non-nutrient agar with *Escherichia coli* heat-inactivated

^c Culture in proteose-peptone-yeast extract medium (Visvesvara and Balamuth, 1975). Positivity observed after 10 days of incubation

^d snPCR targeting the ASA-S1 fragment of 18S rRNA (Dhivya et al. 2007)

Table 3 Results of culture and snPCR for corneal scraping from patients with clinical signs of *Acanthamoeba* keratitis

Sample number	Corneal scraping	
	Culture ^a	snPCR ^b
1	–	+
2	+	+
3	–	+
4	+	+
5	+	+
6	–	+
7	–	+
8	+	+
9	–	–
10	–	–
Total	4/10	8/10

+ positivity, – negativity

^a Culture in soy-agar with *Escherichia coli* (Costa et al. 2010)

^b snPCR targeting the ASA-S1 fragment of 18S rRNA (Dhivya et al. 2007)

Trophozoites and cysts from cultures were used in the initial experiments to determine an efficient DNA extraction technique, including the evaluation of a protocol of alkaline lysis to obtain DNA from *Entamoeba histolytica* and *E. dispar* in feces (Vianna et al. 2009). The protocol was adapted and tested on the assumption that lysis with NaOH could facilitate breaking the cyst wall and releasing the DNA from cysts. The method seems to be relatively efficient for trophozoites, since two out of three repetitions using one and five cells resulted in positivity with snPCR. However, no sign of amplification was observed in snPCR when 10 or fewer cysts were used. These results confirmed the already known resistance of *Acanthamoeba* cysts to DNA extraction, which seems to be related to the resistance of the cyst wall as well as the presence of high-density bodies in the cyst nucleus. These bodies are consisted of core protein condensed by genetic material that can interfere with the DNA denaturation (Goldschmidt et al. 2008; Lasman 1977).

When the extraction kit was used, it was possible to detect 1, 5, and 10 trophozoites or cysts by snPCR in one or more of the triplicate samples. A possible explanation for the higher efficiency of the kit is the use of proteinase K, which could increase the DNA available for the binding to primers and for polymerase action (Goldschmidt et al. 2008). The possibility of false-positive detection as a result of contamination (amplicons) was prevented by careful handling during the procedures and monitoring with control samples, which were all negative.

To obtain corneal scrapings and biopsy for evaluating the molecular technique and to compare it to culture methods, *Acanthamoeba* keratitis was induced in a rat model. The

intrastromal inoculation resulted in lesions of variable grades in all the six animals tested. Although intrastromal inoculation did not replicate the most frequent source of contamination in humans, it provided a higher infection rate, compared to procedures like corneal scratching and the wearing of contaminated contact lenses (Ren and Wu 2010). Thus, the rat model for keratitis could be a good alternative for testing diagnostic procedures applicable to human patients.

Culture in agar plates with *Escherichia coli* is the usual method of diagnosis of *Acanthamoeba* in corneal samples (Siddiqui and Khan 2012). In this study, this method was compared to seeding in PYG medium and seemed to be slightly superior for diagnostic purposes. As the agar plate method is simpler and uses a lower number of components, it was considered the best choice in terms cost-benefit. Furthermore, the higher number of nutrients in PYG could, in some instances, stimulate the growth of organisms other than *Acanthamoeba* in coinfection cases, masking the results.

Comparing the corneal materials, biopsy was clearly superior to corneal scraping regarding the positivity in culture, which can be explained by the higher number of parasites obtained in biopsy. It is known that biopsy is the indicated procedure in cases when the microbial agent is located in deeper layers of the cornea or when corneal scrapings are repeatedly negative, since scraping can be insufficient for the isolation (Younger et al. 2012). However, corneal scraping is the chosen procedure in suspected cases of amoebic keratitis, since it is less invasive and easier to perform. In this study, the snPCR was efficient to detect *Acanthamoeba* in corneal scraping in the animal model, showing that a small amount of tissue can be a limitation for culturing, but not for snPCR. Evaluation of human corneal scrapings for diagnosis of *Acanthamoeba* keratitis also confirmed this point. The human samples that were negative in snPCR were also negative in the culture, suggesting that these keratitis cases should be caused by other causes rather than *Acanthamoeba*. Indeed, empiric treatment for *Acanthamoeba* did not result in improvement of the symptoms, and other causes were then attributed for these cases (data not shown).

In conclusion, DNA extraction with Charge Switch® Kit after freeze/thaw process and amplification by snPCR presented here was efficient to detect the low quantities of trophozoites and cysts that may be present in corneal scrapings. The use of the technique in corneal samples of infected animals and its validation to diagnose human-suspected cases indicated that this is a good alternative for diagnosis of *Acanthamoeba* keratitis.

Acknowledgements The authors thank CNPq, Fundação Araucária, and PRPq/UFGM for financial support. We also thank Ophthalmology Sector staff from Cassiano Antônio de Moraes University Hospital for providing the human corneal scrapings used in this study.

Compliance with ethical standards All procedures with animal were conducted under accepted guidelines for the care and use of laboratory animals for research and approved by the Ethical Committee for Animal Experimentation of the Biological Sciences Sector of Federal University of Paraná, Brazil, protocol number 420b.

The use of information on the diagnosis of patients suspected of *Acanthamoeba* keratitis was approved by the Ethical Committee of Health Sciences Center of Federal University of Espírito Santo, Vitória, ES, Brazil (protocol number 006/07), and followed the tenets of Declaration of Helsinki.

References

- Bacon AS, Frazer DG, Dart JKG, Matheson M, Ficker LA, Wright P (1993) A review of 72 consecutive cases of *Acanthamoeba* keratitis, 1984–1992. *Eye* 7:719–725. doi:10.1038/eye.1993.168
- Claerhout I, Goegebuer A, Van Den Broecke C, Kestelyn P (2004) Delay in diagnosis and outcome of *Acanthamoeba* keratitis. *Graefes Arch Clin Exp Ophthalmol* 242:648–653. doi:10.1007/s00417-003-0805-7
- Costa AO, Castro EA, Ferreira GA, Furst C, Crozeta MA, Thomaz-Soccol V (2010) Characterization of *Acanthamoeba* isolates from dust of a public hospital in Curitiba, Paraná, Brazil. *J Eukaryot Microbiol* 57:70–75. doi:10.1111/j.1550-7408.2009.00453.x
- Dart JKG, Saw VPJ, Kilvington S (2009) *Acanthamoeba* keratitis: diagnosis and treatment update 2009. *Am J Ophthalmol* 148:487–499.e2. doi:10.1016/j.ajo.2009.06.009
- Dhivya S, Madhavan HN, Rao CM, Ramchander PV, Therese KL, Malathi J (2007) Comparison of a novel semi-nested polymerase chain reaction (PCR) with a uniplex PCR for the detection of *Acanthamoeba* genome in corneal scrapings. *Parasitol Res* 100:1303–1309. doi:10.1007/s00436-006-0413-7
- Goldschmidt P, Degorge S, Saint-Jean C, Yera 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 Ophthalmol* 92:112–115. doi:10.1136/bjo.2007.125898
- Khaimar K, Tamber GS, Ralevski F, Pilla DR (2011) Comparison of molecular diagnostic methods for the detection of *Acanthamoeba* spp. from clinical specimens submitted for keratitis. *Diagn Microbiol Infect Dis* 70:499–506. doi:10.1016/j.diagmicrobio.2011.03.019
- Khan NA (2001) Pathogenicity, morphology, and differentiation of *Acanthamoeba*. *Curr Microbiol* 43:391–395. doi:10.1007/s002840010325
- Lasman M (1977) Light and electron microscopic observations on encystment of *Acanthamoeba palestinensis*, Reich. *J Protozool* 24:244–248
- Laummaunwai P, Ruangjirachupom W, Boonmars T (2012) A simple PCR condition for detection of a single cyst of *Acanthamoeba* species. *Parasitol Res* 110:1569–1572. doi:10.1007/s00436-011-2662-3
- Marciano-Cabral F, Cabral G (2003) *Acanthamoeba* spp. as agents of disease in humans. *Clin Microbiol Rev* 16:273–307. doi:10.1128/CMR.16.2.273-307.2003
- Maycock NJR, Jayaswal R, Ed F (2016) Update on *Acanthamoeba* Keratitis: diagnosis, treatment and outcomes. *Cornea* 35:713–720. doi:10.1097/ICO.0000000000000804
- Neff R, Ray S, Benton W, Wilborn M (1964) Induction of synchronous encystment (differentiation) in *Acanthamoeba* sp. *Methods Cell Physiol* 55–83
- Radford CF, Minassian DC, Dart JKG (2002) *Acanthamoeba* keratitis in England and Wales: incidence, outcome, and risk factors. *Br J Ophthalmol* 86:536–542
- Ren M, Wu X (2010) Evaluation of three different methods to establish animal models of *Acanthamoeba* keratitis. *Yonsei Med J* 51:121–127. doi:10.3349/ymj.2010.51.1.121
- Schroeder JM, Booton GC, Hay J, Niszl IA, Seal DV, Markus MB, Fuerst PA, Byers TJ (2001) Use of subgenomic 18S ribosomal DNA PCR and sequencing for genus and genotype identification of acanthamoebae from humans with keratitis and from sewage sludge. *J Clin Microbiol* 39:1903–1911. doi:10.1128/JCM.39.5.1903-1911.2001
- Schuster FL, Visvesvara GS (2004) Amebae and ciliated protozoa as causal agents of waterborne zoonotic disease. *Vet Parasitol* 126:91–120. doi:10.1016/j.vetpar.2004.09.019
- Sharma S, Garg P, Rao GN (2000) Patient characteristics, diagnosis, and treatment of non-contact lens related *Acanthamoeba* keratitis. *Br J Ophthalmol* 84:1103–1108
- Siddiqui, Khan (2012) Biology and pathogenesis of *Acanthamoeba*. *Parasites & Vectors* 2012 5:6. doi:10.1186/1756-3305-5-6
- Sun X, Zhang Y, Li R, Wang Z, Luo S, Gao M, Deng S, Chen W, Jin X (2006) *Acanthamoeba* keratitis: clinical characteristics and management. *Ophthalmology* 113:412–416. doi:10.1016/j.optha.2005.10.041
- Thompson PP, Kowalski RP, Shanks RMQ, Gordon YJ (2008) Validation of real-time PCR for laboratory diagnosis of *Acanthamoeba* keratitis. *J Clin Microbiol* 46:3232–3236. doi:10.1128/JCM.00908-08
- Vianna EN, Costa JO, Santos CKS, Cury MC, Silva EF, Costa AO, Gomes MA (2009) An alternative method for DNA extraction and PCR identification of *Entamoeba histolytica* and *E. dispar* in fecal samples. *Parasitology* 136:765. doi:10.1017/S0031182009006167
- Visvesvara GS, Balamuth W (1975) Comparative studies on related free-living and pathogenic amebae with special reference to *Acanthamoeba*. *J Protozool* 22:245–256
- Yera H, Zamfir O, Bourcier T, Viscogliosi E, Noel C, 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* 26:221–224. doi:10.1007/s10096-007-0268-6
- Younger JR, Johnson RD, Holland GN, Page JP, Nepomuceno RL, Glasgow BJ, Aldave AJ, Yu F, Litak J, Mondino BJ, UCLA Cornea Service (2012) Microbiologic and histopathologic assessment of corneal biopsies in the evaluation of microbial keratitis. *Am J Ophthalmol* 154:512–519.e2. doi:10.1016/j.ajo.2012.03.014