Diagnostic Microbiology and Infectious Disease 74 (2012) 137–141

Contents lists available at SciVerse ScienceDirect

Diagnostic Microbiology and Infectious Disease

journal homepage: www.elsevier.com/locate/diagmicrobio

Parasitology

Rapid detection and simultaneous molecular profile characterization of *Acanthamoeba* infections☆,☆☆

Pablo Goldschmidt [a,*], Sandrine Degorge [a], Djida Benallaoua [a], Laurence Batellier [a], David Di Cave [b], Christine Chaumeil [a]

[a] Laboratoire du Centre Hospitalier National d'Ophtalmologie des Quinze-Vingts 28, rue de Charenton 75012 Paris, France
[b] Department of Public Health and Cell Biology, University of Rome Tor Vergata, Rome, Italy

ARTICLE INFO

Article history:
Received 19 March 2012
Accepted 5 June 2012
Available online 20 July 2012

Keywords:
Acanthamoeba
Detection
High-resolution melting real-time PCR
Keratitis
Genotype

ABSTRACT

Diagnosis of *Acanthamoeba* by microscopic examination, culture, and polymerase chain reactions (PCRs) has several limitations (sensitivity, specificity, lack of detection of several strains, cost of testing for discrimination among strains). We developed a new high-resolution melting real-time PCR (HRM) to detect and characterize *Acanthamoeba* infections. HRM performances were evaluated with strains from the American Type Culture Collection (ATCC) and with 20 corneal scrapings. The DNA extracted from specimens were amplified, detected, and characterized in 1 run using 2 original primers diluted in a solution containing an intercalating dye. Detection and molecular characterization of *Acanthamoeba* infections could be achieved in less than 2.5 h with a dramatic reduction in cost of reactants (postamplification procedures and radioactive or fluorescent-labeled molecular probes were unnecessary). HRM detection limits were 0.1 cyst/μL or less (including genotypes T5 and T11), and its sensitivity and specificity were higher than other molecular tests. For the tested strains from the ATCC, the HRM drafted 4 different profiles: Type I (genotypes T2 and T4), Type II (T5 and T7), Type III (T8), and Type IV (T1, T3, T6, T9, T11, T12, and T13).

1. Introduction

Acanthamoebae are protozoa found in the soil, aquatic environment, and air. They are generally associated with eye diseases, although general infections (mostly brain and skin) were reported also. The infestations with *Acanthamoeba* are related to poor hygiene, inhalation of contaminated water, immunosuppression, corneal lens use, and corneal injuries (Lorenzo-Morales et al., 2010; Marciano Cabral, 2003).

To reduce morbidity, sensitive, specific, and inexpensive tests are necessary to administer therapeutic agents at the onset of symptoms. However, the direct smear analysis of corneal scrapings (Gram, May-Grünwald-Giemsa pH 7.4 staining) produces results rapidly, but misdiagnoses almost 60% of clinical cases (Behets et al., 2006; Yera et al., 2007).

The detection of *Acanthamoebae* by polymerase chain reactions (PCR) dramatically enhanced diagnosis performances. The tests targeting the *Acanthamoeba* nuclear small subunit ribosomal 18S rRNA gene detected the equivalent of 1 protozoa per sample (Clarke and Niederkorn, 2006; Gatti et al., 2010; Marciano Cabral, 2003), but confirmation of the amplicon specificities required a series of additional procedures (agarose gel electrophoresis, hybridisation, and/or molecular sequencing), for which the opening of tubes with amplified material enhanced cross-contamination risks (Boggild et al., 2009; Goldschmidt et al., 2009a; Yera et al., 2007).

The reduction in postamplification handling was possible using a real-time technology (real-time PCR [rtPCR]), for which the amplicons produced during the rtPCR could be detected in closed tubes by means of 2 different strategies: a) fluorophore-labeled TaqMan probes or b) the intercalating fluorophore SYBR-green. Nevertheless, the SYBR-green rtPCR did not fulfill the expectations for diagnosis because traces of residual double-stranded DNA from other agents found in the DNA extracted from the samples may bind the fluorophore and trigger nonspecific positive signals. Here, detecting fluorescent signals required the addition of postamplification procedures (agarose gel electrophoresis or sequencing) (Behets et al., 2006; Boggild et al., 2009) to determine species or genotypes. The use of labeled TaqMan® probes (Qvarnstrom et al., 2006; Yera et al., 2007) instead of SYBR-green improved rtPCR specificity but did not detect all the genotypes. In addition, TaqMan rtPCRs were unable to provide group or genotype data (Da Rocha-Azevedo et al., 2009; Qvarnstrom et al., 2006). We reported a broad-spectrum rtPCR performed directly from clinical or

☆ Conflict of interests: none for all authors.
☆☆ Funding sources: This work was supported exclusively by intramural funds of the Laboratoire d'Ophtalmobiologie du Centre Hospitalier National d'Ophtalmologie des Quinze-Vingts, Paris, France.
* Corresponding author. Tel.: +33-1-40-02-16-94; fax: +33-1-40-02-16-99.
E-mail addresses: pablogol@aol.com, goldschmidt@quinze-vingts.fr (P. Goldschmidt).

0732-8893/$ – see front matter © 2012 Elsevier Inc. All rights reserved.
doi:10.1016/j.diagmicrobio.2012.06.001

environmental samples (targeting a highly conserved mitochondrial gene) that made possible the detection of all the available American Type Culture Collection (ATCC) strains (0.1 cyst/μL of sample). Like other TaqMan rtPCRs, the broad-spectrum test that required expensive fluorophore-labeled probes was not informative on groups, species, types, or genotypes (Goldschmidt et al., 2009b).

The high-resolution melting real-time PCR technology (HRM) was developed for the fine assessment of DNA sequence variations (Monis et al., 2005; Reed et al., 2007) using improved fluorescent DNA-binding dyes. In this study, we developed a new molecular strategy based on HRM that should a) detect in 1 run the equivalent of 1 cyst of *Acanthamoeba* or less per sample; b) produce results without molecular probes based on the dye-melting derivative curve analysis with no need for gel electrophoresis, hybridizations, or immunoenzymatic assays; and c) draft different profiles for different species according to the guanine cytosine content and to the size of the amplicons neosynthesised during the nucleic-acid amplification process

2. Material and methods

HRM sensitivity, specificity, linearity, and detection limits were first assessed with strains of *Acanthamoeba* purchased from LGC Promochem (Molsheim, France; an ATCC partner) (*A. comandoni* [ref. 30135], *A. palestinensis* [ref. 50708], *A.* sp. [ref. 50655], *A. palestinensis* [ref. 30870], *A. griffini* [ref. 30731], *A. castellanii* [ref. 50373], *A. lenticulata* [ref. 30841], *A. astronyxis* [ref. 30137], *A. tubiashi* [ref. 30867], and *A. hatchetti*). *Acanthamoeba* were cultured and maintained according to the procedures indicated by the ATCC for each strain (Da Rocha-Azevedo et al., 2009; Marciano Cabral, 2003).

Investigations were conducted according to the principles expressed in the Declaration of Helsinki (http://www.wma.net/en/30publications/10policies/b3/index.html) and followed the requirements for approval by the Institutional Review Board of the Centre Hospitalier National des Quinze-Vingts (CHNO), Ministry of Public Health, Paris, France. For each sample, forms with written consent drafted according to the requirements of the CHNO Review Board and the National Health Authorities were sent to the laboratory after being double checked, validated, and signed by the physician in charge of the sampling.

After rinsing fluorescein and topical anesthetics from the eye surface, ophthalmologists performed deep scraping of the cornea with sterile stainless steel blades in order to obtain materials from patients presenting with corneal ulcers and requiring microbiological diagnosis (Goldschmidt et al., 2006, 2008). Samples were divided into aliquots and tested masked for the following:

a) Direct microscopic examination (specimens were fixed and stained with Giemsa [pH 7.4] and confirmed by Grocott's methenamine silver reaction);
b) Culture for aerobic and anaerobic bacteria and fungi. Isolates were phenotypically characterized by conventional tests (Chromagar *Candida* BR [ref. 257480], Becton Dickinson, Rungis, France; API 20AUX [ref. 20210], bioMérieux, Craponne, France; API *Candida*, bioMérieux; and Lactophenol Blue [ref. 363060–0125], RAL Advanced Chromatic, Martillac, France). Aliquots of each scraping were discharged into flasks with 5 mL of 0.9% sodium chloride and *Escherichia coli* suspensions and incubated up to 30 days before being discarded as culture negative (Da Rocha-Azevedo et al., 2009; Marciano Cabral, 2003; Yera et al., 2007);
c) DNA extraction for diagnosis of *Herpesviridae* by real-time PCR (Goldschmidt et al., 2006; Van Doornum et al., 2003) and of *Acanthamoeba* (Behets et al., 2006; Goldschmidt et al., 2008).

DNA was extracted in a vertical safety laminar flow cabinet. To monitor the yields of extraction and the PCR inhibitors, the internal control (IC) consisting of a virus preparation of seal herpes virus (Van Doornum, Department of Virology Erasmus MC, Rotterdam, The Netherlands) was added before extraction and 200 μL was used for the extraction on a MagNA Pure compact instrument (Roche, Meylan, France) (Goldschmidt et al., 2008; Van Doornum et al., 2003). DNA extracts were tested by 2 rtPCRs targeting different genes (Behets et al., 2006; Goldschmidt et al., 2009a, b; Qvarnstrom et al., 2006) and by HRM. The primers selected for HRM bracket significant polymorphisms in the mitochondrion gene (gi|562028|gb|U12386.1|ACU12386, U12386, U12386.1GI: 562028). Forward and reverse HRM primer sequences are 5′GCAGTCGCGGTAATACGA and 5′ ACCACCTACGCACCCTTTACA, respectively.

Kits prepared for HRM consist of 2 tubes: the first for detection, semiquantification, and molecular profile characterization of *Acanthamoeba* (10 μL of MeltDoctor® HRM Master Mix [ABI ref. 4415440, Applied Biosystems, France] and 1 μL each of the forward and the reverse primers, each at 300 nmol/L final concentration); and the second for amplification of the IC (seal herpes). HRM was performed by introducing 10 μL of the DNA extract in the tube for *Acanthamoeba* detection and 10 μL in the tube for the IC.

HRM amplicon production and size were automatically assessed with the ABI 7500 upgraded equipment. The amplification program consisted in one cycle of 10 min at 95 °C, followed by 55 cycles (15 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C). The HRM dye melting curve was obtained by heat denaturation (95 °C for 15 s, cooling to 50 °C for 1 min, and a temperature increase until 60 °C for 15 s with a 2.2 °C/s ramp rate). The difference plot was used for amplicon analysis, and the specificity of the assay was guaranteed by the right peak and the additional melting analysis. The first derivative of the melting curves and the "melt peaks" (Tm) allowed establishing comparisons between clinical isolate profiles and the referenced strains from the ATCC. Samples with fluorescence of less than 100% of the maximum were excluded from the analysis. The internal IC was amplified in an independent real-time PCR run (Goldschmidt et al., 2008, 2009b; Van Doornum et al., 2003).

HRM specificity was assessed with DNA extracts obtained by mixing 100 μL of suspensions of 10^6/mL of human fibroblasts or 10^6 CFU/mL of different bacteria or fungi or 10^6 PFU/mL of herpes simplex virus (HSV) type 1 and type 2, or varicella zoster virus (VZV) or *Adenovirus* type 8 with or without 20 *Acanthamoeba* cysts.

Genotyping of *Acanthamoeba* was carried out by amplification and analysis of the ASA.S1 region of the 18S rRNA gene using the genus-specific primers JDP1 and JDP2 (Goldschmidt et al., 2009b; Marciano Cabral, 2003). Sequences were aligned with a standard set of sequences, and the analysis of the nearly complete Rns genes allowed genotype classification. Bacteria, fungi, or viruses were characterized with kits validated for clinical diagnosis. Clinical samples were read masked by 2 trained technicians and confirmed by 2 members of the medical staff of the clinical laboratory.

3. Results

The selected primers detected *Acanthamoeba* with a linear range spanning from 10^5 to at least 10^{-1} cyst/mL. The interassay reproducibility coefficient variation was less than 10% among HRM runs for each dilution. The HRM coefficient of variation for the interassay reproducibility in the complete linear range of detection (10^5 to 1 cyst/mL) for 5 runs was inferior to 10%.

The reactants, disposable material, and environmental controls did not produce signals that could have interfered with the interpretation of results. Human cells and several human pathogens did not interfere with HRM (*Staphylococcus epidermidis* and *aureus*, *Streptococcus pneumoniae*, *Serratia marcescens*, *E. coli*, *Pseudomonas acnes*, *P. aeruginosa*, *Candida albicans*, *Fusarium* sp., *Aspergillus* sp., *HSV-1 and -2*, VZV, and *Adenovirus* type 8).

As shown in Table 1, HRM detected all the strains (detection limits: 10^{-1} cysts/μL or less) and drafted simultaneously 4 different consistent profiles: Profile I for genotypes T2 and T4; Profile II for T5

Table 1
Comparison of *Acanthamoeba* nuclear 18S ribosomal RNA genotypes with high-resolution melting analysis (HRM).

Strain	Genotype	Result (1 cyst/reaction)	HRM
A.castellani	T4	Positive	Profile 1
A. palestinensis	T2	Positive	Profile 1
A. palestinensis 50708	T2	Positive	Profile 1
A. astronyxis	T7	Positive	Profile 2
A. lenticulata	T5	Positive	Profile 2
A. tubiashi	T8	Positive	Profile 3
A. comandoni	T9	Positive	Profile 4
A. griffini	T3	Positive	Profile 4
A. hatchetti	T11	Positive	Profile 4
A. sp*	T1	Positive	Profile 4
A. sp*	T6	Positive	Profile 4
A. sp*	T13	Positive	Profile 4
A. sp*	T14	Positive	Profile 4
A. healyi	T12	Positive	Profile 4

* Isolates from *Acanthamoeba* keratitis tested masked (5 different experiments for each).

and T7; Profile III for T8; and Profile IV for genotypes T1, T3, T6, T9, T11, T12, T13, and T14 (Table 1). Compared to controls, the Ct values for the IC were never delayed for more than 1.5 cycles.

In samples obtained from patients with *Acanthamoeba* keratitis, the direct microscopic examination detected cysts in 50% and 60% which were *Acanthamoeba* culture positive. The time for culture positive ranged between 2 and 4 weeks.

For each sample, the results were validated only if the Ct value obtained for the IC compared to the blank was not delayed by more than 1.5 cycles. The Ct values obtained here for the ICs indicate that the yields of extraction of DNA were satisfactory and that the potential PCR inhibitors were eliminated. As shown in Table 2, HRM was negative for all the samples testing negative for *Acanthamoeba* culture, negative for *Acanthamoeba* TaqMan rtPCRs targeting the ribosomal (Qvarnstrom et al., 2006; Rivière et al., 2006) or the mitochondrial gene (Goldschmidt et al., 2009a), and negative for the SYBR-green rtPCRs.

The 2 SYBR-green rtPCRs were positive for all the samples from *Acanthamoeba* keratitis (samples 1 to 10), but the signal analysis did not allow establishing molecular profiles. SYBR-Green specificity was 85%. Sensitivity and specificity for the TaqMan rtPCR targeting the ribosome (Qvarnstrom et al., 2006) were 90% for both (negative results for genotype T5 and very weak signals for genotypes T1 and T3). The ribosome-targeted rtPCR repeatedly produced false-positive results for samples containing *S. lexinton*, *Clostridium sporulans*, and *P. acnes*. Sensitivity and specificity for the TaqMan rtPCR targeting the mitochondrion (Goldschmidt et al., 2009a, b) were both 100% (Table 2).

Table 3 presents the HRM profiles compared to ribosome gene sequencing. Clinical isolates here were largely (70%) T4 and T3 as previously reported for AK (Booton et al., 2005; Lorenzo-Morales et al., 2010; Marciano-Cabral, 2009) and 10% were genotype T5 and 10% were T11. Eight of 10 HRM profiles correspond to what was expected from ribosome sequencing, but 2 isolates characterized as genotype T4 showed unexpected type I HRM profile.

4. Discussion

Mixing the DNA extracted from samples with a solution containing primers from a selected sequence and 1 intercalating dye that stoichiometric binds reversibly to the neoformed double-stranded DNA during amplification makes it possible to conduct an automatic melting analysis to detect *Acanthamoeba* that provides 4 reproducible signatures for the different genotypes. The detection of *Acanthamoeba* and their molecular characterization are performed by obviating gel electrophoresis, hybridizations, immunoassays, etc., because the automatic melting analysis amplified with 1 set of primers suited consistently to discriminate among 4 *Acanthamoeba* mitochondrial

Table 2
Direct microscopic examination, culture, real-time PCR, and HRM performances on samples obtained from patients with keratitis.

No.	Test						
	Direct microscopic examination of stained scrapings	Culture (time for positivity, weeks)	TaqMan real-time PCR[a,c]	TaqMan real-time PCR[a,d]	SYBR Green real-time PCR[b,d]	HRM	Genotype+
	Results						
1	Positive	Positive, 2	Positive	Positive**	Positive	Positive	T4
2	Positive	Negative	Positive ##	Positive**	Positive	Positive	T11
3	Negative	Negative	Positive ##	Positive**	Positive	Positive	T3
4	Negative	Positive, 3	Negative	Positive**	Positive	Positive	T5
5	Positive	Positive, 3	Positive	Positive**	Positive	Positive	T4
6	Positive	Negative	Positive	Positive**	Positive	Positive	T4
7	Negative	Positive,4	Positive	Positive**	Positive	Positive	T4
8	Positive	Positive, 2	Positive	Positive**	Positive	Positive	T4
9	Negative	Negative	Positive	Positive**	Positive	Positive	T4
10	Negative	Positive;3	Positive	Positive**	Positive	Positive	T4
11	Negative	*P. aeruginosa*	Negative	Negative	Negative	Negative	
12	Negative	*S. aureus*	Negative	Negative	Negative	Negative	
13	Negative	*S. lexinton*	False positive ##	Negative	False positive ##	Negative	
14	Negative	*S. pyogenes*	Negative	Negative	Negative	Negative	
15	Negative	*P. aeruginosa*	Negative	Negative	Negative	Negative	
16	Negative	*A. freundii*	Negative	Negative	Negative	Negative	
17	Negative	*C. sporulans*	Negative	Negative	False positive ##	Negative	
18	Negative	*S. marcescens*	Negative	Negative	Negative	Negative	
19	Negative	*P. acnes* +*S. lexinton*	False positive ##	Negative	False positive ##	Negative	
20	Negative	*E. coli*	Negative	Negative	Negative	Negative	

= Weak positive signals (40 ± 2 cycles); positive = at least 1 image evoking cysts (read by 2 technicians and by 2 members of the medical staff) or positive signal; negative = negative signal; ** = Ct < 35; + = nuclear 18S ribosomal RNA gene sequencing.
[a] Internal control Ct values ranged between 31.3 and 33.1.
[b] Peak at 78 °C.
[c] Qvarnstrom et al. (2006).
[d] Goldschmidt et al. (2009a).

Table 3
Genotyping analysis of corneal isolates (ribosomal sequences)[a] compared to HRM.[b]

Clinical isolate genotype[a]	HRM profile[b]	Expected profile according to sequencing
T4	4	1[c]
T11	4	4
T3	4	4
T5	2	2
T4	1	1
T4	4	1[c]
T3	4	4
T4	1	1
T4	1	1
T4	1	1

[a] The amplification of the ASA.S1 region of the 18S rRNA gene using the primers JDP1 and JDP2 was followed by sequence reactions, capillary electrophoresis, and molecular alignments (Booton et al., 2009, Goldschmidt et al., 2009b).
[b] HRM molecular profiles were directly obtained from the corneal samples.
[c] Discordances between HRM mitochondrial sequence analysis and ribosomal molecular sequencing.

gene groups. The classic PCRs and the TaqMan rtPCRs are unable to detect all the *Acanthamoeba* species (Boggild et al., 2009; Goldschmidt et al., 2009a; Ledee et al., 2003; Qvarnstrom et al., 2006), and, therefore, additional procedures should be carried out to avoid false conclusions. On the other hand, SYBR rtPCRs have a limited predictive value because false-positive signals are repeatedly obtained with residual nucleic acids present in the DNA extracts and false-negative results were reported (Taq-DNA polymerase inhibition by the fluorophore) (Boggild et al., 2009; Monis et al., 2005; Reed et al., 2007).

The new HRM a) detects at least 0.1 cyst/mL of all the tested strains of *Acanthamoeba*; b) confirms diagnosis directly from clinical samples; c) assesses simultaneously the yields of DNA extraction and the PCR inhibitors; d) drafts 4 different molecular profiles; e) is performed without molecular probes (radioactive or nonradioactive); and f) allows results with molecular characterizations to be obtained in less than 2.5 h after DNA extraction; f) is not modified by high loads of bacteria, fungi, herpes simplex viruses, adenoviruses, or human cells. HRM allowed corneal samples to be readily assayed after DNA extraction without interference from DNA sequences that may be found in the specimen. For the series of 20 corneal scrapings tested here, HRM detecting capacities and specificity were higher than culture and all other nucleic-acid amplification techniques (Behets et al., 2006; Da Rocha-Azevedo et al., 2009; Rivière et al., 2006). The loop-mediated isothermal amplification (LAMP) is a method that does not require thermocyclers and DNA purification procedures. LAMP was recently developed for the diagnosis of *Trypanosoma* spp., *Cryptosporidium* spp., *Theileria*, canine and equine piroplasmosis, *Toxoplasma gondii, Giardia duodenalis*, *Microsporidia*, *Taenia* sp., and *Plasmodia* (Karanis and Ongerth, 2009) (Baldursson and Karanis, 2011). Further investigations should be conducted to compare HRM and LAMP performances for the detection, quantification, and characterization of *Acanthamoeba* infections.

Major limitations were reported for epidemiologic and physiopathology studies, for which the taxonomy of *Acanthamoeba* was based on cyst morphology and trophozoite size and shape. In fact, protozoa morphology changes according to culture conditions and, therefore, further phylogenetic analysis was conducted according to the nuclear 18S ribosomal RNA gene (Rns or 18S rDNA) sequence that considered that a 5% dissimilarity value separates genotypes (Goldschmidt et al., 2009b; Ledee et al., 2003; Marciano Cabral, 2003). The Rns trees compared with those obtained by sequencing the mitochondrial small subunit rRNA gene (Rns or 16S rDNA) failed to distinguish between genotypes T3 and T11. HRM (without electrophoresis, hybridization, sequencing reactions, or any additional laborious postamplification manipulation) drafted similar profiles (HRM profile type IV) for samples containing the Rns genotypes T3 and T11.

The ribosomal 18S rDNA Rns phylogenetic relationships identified a major clade designated T4, which included 53 different strains having 22 different sequences (Marciano-Cabral, 2009; Booton et al., 2009). In the present study, 2 clinical samples characterized by Rns sequencing as T4 produced an unexpected HRM type IV profile (instead of type I). These results may confirm previous reports suggesting that strains from the T4 clade could be distinguished with a higher level of significance for trees built according to the Rns mitochondria-associated sequences (Ledee et al., 2003). In the present study, the genotype T4 was detected in 70% of keratitis, from which only 50% produced the expected T4 HRM profile. HRM may therefore provide relevant additional information for the comprehensive classification of protozoa (Ledee et al., 2003; Marciano Cabral, 2003) and confirm hypothesis suggesting that the named taxa based on 18S rDNA Rns phylogenetic relationship genotyping are not monophyletic entities. In previous 18S rDNA-based reports, the genotype T4 was predominant (~85%) (Boggild et al., 2009; Booton et al., 2009; Ledee et al., 2003; Lorenzo-Morales et al., 2010; Schroeder et al., 2001; Zhao et al., 2010). However, in Greece, the genotypes T2, T3, T5, T6, and T11 have been identified (Spanakos et al., 2006) and in Slovakia and the Czech Republic the genotypes T15, T4, and T3 were detected in AK (Nagyová et al., 2010). In Italy, 25% of the global *Acanthamoeba* keratitis isolates were identified as T15 (Di Cave et al., 2009).

The *Acanthamoeba* cultures require time, rely on the ability of the protozoa to grow ex vivo, are time consuming, and may become positive late in the course of the infection. Great improvement was obtained using TaqMan rtPCR tests but they are unable to detect strains from genotype T5 (Qvarnstrom et al., 2006) and *A. astronyxis* (Da Rocha-Azevedo et al., 2009; Qvarnstrom et al., 2006). Moreover, the cost for the numerous TaqMan probes and for the postamplification procedures required for characterizing *Acanthamoeba* is high (Gatti et al., 2010; Goldschmidt et al., 2009b; Khairnar et al., 2011; Rivera and Adao, 2009; Schroeder et al., 2001; Yera et al., 2007).

As shown in Table 3, HRM profiles I and IV have the potential to cause keratitis (samples from patients presenting with *Acanthamoeba* keratitis). However, the sample size of this study is too small to state general conclusions, and, therefore, further investigations in different settings with a larger series of samples are necessary to confirm the association of each of the 4 HRM profiles with pathology, tropism, and environmental distribution.

In conclusion, HRM detects *Acanthamoeba* (including genotypes T5 and T11) and drafts molecular profiles with higher sensitivity than culture and other molecular tools. It has the relevant advantage of minimizing cross-contamination risks because the amplification procedures and the signal detection and the DNA melting analyses are carried out in closed tubes. In addition, HRM minimizes the risks for false-negative conclusions because the yields of extraction of DNA and the PCR inhibitors are systematically monitored. The cost for reactants for testing 1 DNA extract (detection and molecular characterization of *Acanthamoeba*) could be reduced to less than US $2.00 because HRM can be carried out with the equipment routinely used in laboratories for real-time PCR (upgrading the software required) with no need for molecular probes, gels, restriction enzymes, or any additional equipment for sophisticated postamplification procedures. Future analyses should generate reproducible melt curves over time in different settings to build reference databases to store HRM calculations and shapes of the melting profiles for each family or species in order to be challenged against the results of future testing. On the above, larger trials are necessary to confirm the usefulness of HRM for environmental studies and disease management (Baldursson and Karanis, 2011; Karanis and Ongerth, 2009).

Acknowledgments

The authors thank the Centre de Ressources Biologiques du Centre Hospitalier National d'Ophtalmologie des Quinze-Vingts, Paris, France, for providing the biological samples.

References

Baldursson S, Karanis P. Waterborne transmission of protozoan parasites: review of worldwide outbreaks—an update 2004–2010. Water Res 2011;15:6603–14.

Behets J, Declerck P, Delaedt Y, et al. Quantitative detection and differentiation of free-living amoeba species using SYBR green-based real-time PCR melting curve analysis. Curr Microbiol 2006;53:506–9.

Boggild AK, Martin DS, Lee TY, et al. Laboratory diagnosis of amoebic keratitis: comparison of four diagnostic methods for different types of clinical specimens. J Clin Microbiol 2009;47:1314–8.

Booton GC, Visvesvara GS, Byers TJ, et al. Identification and distribution of *Acanthamoeba* species genotypes associated with nonkeratitis infections. J Clin Microbiol 2005;43:1689–93.

Booton G, Joslin C, Shoff M, et al. Genotypic identification of *Acanthamoeba* sp. isolates associated with an outbreak of acanthamoeba keratitis. Cornea 2009;28:673–6.

Clarke DW, Niederkorn JY. The pathophysiology of *Acanthamoeba* keratitis. Trends Parasitol 2006;22:175–80.

da Rocha-Azevedo B, Tanowitz HB, Marciano-Cabral F. Diagnosis of infections caused by pathogenic free-living amoebae. Interdiscip Perspect Infect Dis 2009;2009:251406.

Di Cave D, Monno R, Bottalico P, Guerriero, et al. *Acanthamoeba* T4 and T15 genotypes associated with keratitis infections in Italy. Eur J Clin Microbiol Infect Dis 2009;28: 607–12.

Gatti S, Rama P, Matuska S, et al. Isolation and genotyping of *Acanthamoeba* isolates from corneal infections in Italy. J Med Microbiol 2010;12:20705732.

Goldschmidt P, Rostane H, Saint-Jean C, et al. Effects of topical anaesthetics and fluorescein on the real-time PCR used for the diagnosis of herpesviruses and *Acanthamoeba* keratitis. Br J Ophthalmol 2006;90:1354–6.

Goldschmidt P, Degorge S, Saint-Jean C, et al. Resistance of *Acanthamoeba* to classic DNA extraction methods used for the diagnosis of corneal infections. Br J Ophthalmol 2008;92:112–5.

Goldschmidt P, Dardé M, Yera H, et al. Rapid detection and characterization of pathogens by molecular techniques. In: Liu Dongyou, editor. Molecular detection of foodborne pathogens; Chapter 45: *Acanthamoeba*. CRC Press1420076434; 2009a.

Goldschmidt P, Degorge S, Benallaoua D, et al. New tool for the simultaneous detection of 10 different genotypes of *Acanthamoeba* available from the American Type Culture Collection. Br J Ophthalmol 2009b;93:1096–100.

Karanis P, Ongerth J. LAMP: a powerful and flexible tool for monitoring microbial pathogens. Trends Parasitol 2009;25:498–9.

Khairnar K, Tamber G, Ralevski F, Pillai D. Comparison of molecular diagnostic methods for the detection of *Acanthamoeba* spp. from clinical specimens submitted for keratitis. Diagn Microbiol Infect Dis 2011;70:499–506.

Ledee DR, Booton GC, Awwad MH, et al. Advantages of using mitochondrial 16S rDNA sequences to classify clinical isolates of *Acanthamoeba*. Invest Ophthalmol Vis Sci 2003;44:1142–9.

Lorenzo-Morales J, Marciano-Cabral F, Lindo JF, et al. Pathogenicity of amoebae. Exp Parasitol 2010;126:2–3.

Marciano Cabral F. *Acanthamoeba* spp. as agents of disease in humans. Clin Microbiol Rev 2003;16:273–307.

Marciano-Cabral F. Free-living amoebae as agents of human infection. J Infect Dis 2009;15(199):1104–6.

Monis P, Giglio S, Saint C. Comparison of SYT09 and SYBR Green I for real-time polymerase chain reaction and investigation of the effect of dye concentration on amplification and DNA melting curve analysis. Anal Biochem 2005;340:24–34.

Nagyová V, Nagy A, Timko J. Morphological, physiological and molecular biological characterisation of isolates from first cases of *Acanthamoeba* keratitis in Slovakia. Parasitol Res 2010;106:861–72.

Qvarnstrom Y, Visvesvara G, Sriram R, da Silva A. Multiplex real-time PCR assay for the simultaneous detection of of *Acanthamoeba* spp., *Balamuthia mandrillaris*, and *Naegleria fowleri*. J Clin Microbiol 2006;44:3589–95.

Reed G, Kent J, Wittwer C. High-resolution DNA melting analysis for simple and efficient molecular diagnostics. Pharmacogenomics 2007;8:597–608.

Rivera W, Adao D. 18S ribosomal DNA genotypes of *Acanthamoeba* species isolated from contact lens cases in the Philippines. Parasitol Res 2009;105:1119–24.

Rivière D, Szczebara F, Berjeaud J, et al. Development of a real-time PCR assay for quantification of *Acanthamoeba* trophozoites and cysts. J Microbiol Methods 2006;64:78–83.

Schroeder J, Booton G, Hay J, et al. Use of subgenic 18S ribosomal DNA PCR and sequencing for genus and genotype identification of *Acanthamoeba* from humans with keratitis and from sewage sludge. J Clin Microbiol 2001;39:1903–11.

Spanakos G, Tzanetou K, Miltsakakis D, et al. Genotyping of pathogenic *Acanthamoebae* isolated from clinical samples in Greece: report of a clinical isolate presenting T5 genotype. Parasitol Int 2006;55:147–9.

Van Doornum G, Guldemeester J, Osterhaus ADME, et al. Diagnosing herpesvirus infections by real-time amplification and rapid culture. J Clin Microbiol 2003;41: 2576–80.

Yera H, Zamfir O, Bourcier T, et al. 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 2007;26:221–4.

Zhao G, Sun S, Zhao J, Xie L. Genotyping of *Acanthamoeba* isolates and clinical characteristics of patients with *Acanthamoeba* keratitis in China. J Med Microbiol 2010;59:462–6.