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► **To cite this version:**

Pablo Goldschmidt, Sandrine Degorge, Djida Benallaoua, Cathy Saint-Jean, Laurence Batellier, et al.. New tool for the simultaneous detection of ten genotypes of *Acanthamoeba*. *British Journal of Ophthalmology*, 2009, 93 (8), pp.1096-n/a. 10.1136/bjo.2008.144873 . hal-00477811

**HAL Id: hal-00477811**

**<https://hal.science/hal-00477811>**

Submitted on 30 Apr 2010

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**New tool for the simultaneous detection of ten different genotypes of  
*Acanthamoeba* available from the American Type Culture Collection**

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Competing Interest: None declared

**Key words:**

*Acanthamoeba*, diagnosis, real-time PCR, keratitis

**Abstract****New tool for the simultaneous detection of ten different genotypes of *Acanthamoeba* available from the American Type Culture Collection**

**Background:** *Acanthamoeba keratitis* (AK) is a sight-threatening infection and none of the current diagnosis tests are able to detect in one reaction low levels of the vast majority of strains associated with pathology. The goal of this work was to validate a new tool for the detection of the American Type Cell Collection referenced *Acanthamoeba* monitoring simultaneously DNA extraction yields and PCR inhibitors. Performances were assessed on corneal scrapings. **Methods:** Primers were selected in a region bracketing a 41591 bp of the *A. castellanii* mitochondrion gene. DNA extraction and PCR inhibitors were monitored by adding an internal control (virus). *Acanthamoeba* were detected and quantified by the real time fast duplex TaqMan<sup>®</sup> PCR (f-d-real-t PCR) and negativity confirmed by SYBR Green real time PCR. **Results:** The f-d-real-t PCR detects 0.1 cyst/ $\mu$ l or less of the 10 referenced strains (sensitivity slightly lower for *A. astronyxis*). Bacteria, fungi and Herpesviruses do not cross-react. The specificity and sensitivity of the f-d-real-t PCR were higher than culture and other real-time PCR on 20 keratitis samples. **Conclusion:** The f-d-real t PCR detects in less than 2 h the *Acanthamoeba* strains available from the ATCC with higher sensitivity and specificity than techniques previously reported. Larger trials are necessary to validate its usefulness for disease management and environmental studies.

**Word count: 207**

## **New tool for the simultaneous detection of ten different genotypes of *Acanthamoeba* available from the American Type Culture Collection (ATCC)**

### **Introduction**

*Acanthamoebae* are protozoan found worldwide as free living organisms in the soil, aquatic environment (water, swimming pools, seawater, sewage, etc) and in air.[1-2] In immune compromised subjects *Acanthamoebae* are associated with brain (granulomatous fatal encephalitis), skin and eye infections (chorioretinitis, endophthalmitis).[3-4]

*Acanthamoeba* keratitis (AK) is a sight-threatening infection associated with soft contact lenses, poor hygiene and contaminated water in swimming pools, showers, etc, caused by different genotypes. [5-7] In developing countries AK is mostly associated with corneal injuries. At advanced stages AK often requires corneal transplants or even enucleation.[8]

Rapid conventional laboratory diagnosis for AK can be conducted by direct examination of corneal scrapings (Gram, May-Grünwald-Giemsa pH 7.4 staining); however, it was reported that 60% of the clinical AK are misdiagnosed by direct smear analysis.[2, 5, 9, 10-12] The methods based on nucleic acid amplification techniques (NAATs) enhanced the diagnosis sensitivity. However the techniques requiring manipulations with amplified products increased cross contamination risks by dispersion of DNA sequences.[12-15] The risks for contamination were dramatically reduced by the development of the real-time PCRs, but for none of them the capacity to detect the ATCC referenced strains was reported.[16-19]

The pertinent therapeutic interventions at the onset of signs improve the prognosis and limit the corneal opacities leading to blindness and therefore, highly sensitive and specific diagnosis tools are necessary to administrate on time the efficient treatments for AK.[5, 9, 10] The goal of this work was to design and validate a new NAAT diagnosis strategy with no cross reactivity with mammal, yeast, viral or bacterial genes. It has to assess in one run simultaneously the DNA extraction yields and the Taq polymerase inhibitors. This new test has to detect the equivalent of 0.1 cysts/µl or less of the species available from the ATCC and should be adaptable to different thermocyclers. The sensitivity, specificity and the confirmation of negativity will be validated with clinical samples.

## Material and Methods

### Strains of *Acanthamoeba*

The ATCC's available strains were purchased from its correspondent in France, LGC Prochem. [Tubes were labelled as: *A. comandoni* (ref 30135); *A. palestinensis* (ref 50708); *A. sp* (ref 50655), *A. palestinensis* (ref 30870); *A. griffini* (ref 30731); *A. castellani* (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 specific procedures indicated by the ATCC for each strain. The viability of parasites, the number of trophozoites, the cyst load and the cyst morphology were assessed daily by direct microscopic examination. Quantified viable cultures were processed for DNA extraction.

### AK samples

Samples were obtained in accordance with "Declaration of Helsinki" ([http:// www. wma .net /e/policy/](http://www.wma.net/e/policy/)) from patients with suspicion of AK and from patients presenting with keratitis requiring microbiological diagnosis by ophthalmologists under slit lamp by deep scraping of the cornea with sterile stainless steel blades [20] and sent to the laboratory for conventional culture and real time PCR. Aliquots were frozen and tested blind after addition of 200 µl sterile Phosphate Buffer Solution (PBS). The bacterial, fungal or viral strains from clinical samples were isolated and characterized by routine classic culture tests.

### Primers and probe

The sequences were obtained from the 41591 bp DNA *Acanthamoeba castellanii* mitochondrion gene (gi|562028|gb|U12386.1|ACU12386, complete genome Accession U12386, U12386.1GI:562028) <http://www.ncbi.nlm.nih.gov>. Forward primer: 5'GCAGTCGCGTAATACGA; reverse primer: 5'ACCACCTACGCACCCTTTACA and TaqMan<sup>®</sup> probe: 6-FAM-AGTGT TATTCGCATTGACTGGGTGTAA-TAMRA.

### DNA extraction

Five µl of a whole virus preparation of Seal Herpesvirus (phHV) (gift from van Doornum, Dept. of Virology Erasmus MC, Rotterdam, The Netherlands) were added to 200 µl of each cyst suspension

before extraction (final concentration of 1000 to 2000 viral particles/ ml) to monitor the extraction processes and to assess the potential PCR inhibitors.[21N2] The suspensions were mixed with 200 µl of ATL buffer (Qiagen, France) and 40 µl of Proteinase K (Qiagen) and incubated at 56°C for 10 min. Proteolysis was stopped in a dry plate at 95°C for 10 min and the DNA was extracted with the MagNA Pure<sup>®</sup> Nucleic Acid isolation kit (MagNA Pure LC<sup>®</sup>-Roche) and eluted in 100µl.[22] The extract were cooled immediately after (-20°C) for 5 min and temperature was stabilized for 30 min at 20°C.

### **Real-time TaqMan PCR**

In preliminary assays were determined the optimal conditions (primers and probes, volumes and temperatures) in which at least the equivalent of 0.1 cyst/µl of *Acanthamoeba* genotype T 4 were repeatedly detected. For the duplex fast real-time PCR (f-d-real-t PCR) the differences in the emission wavelength spectrums for each fluorophore bind to probes were > to 15 nanometers. The f-d-real-t PCR was carried out in a final volume of 25 µl containing 2X TaqMan<sup>®</sup> Fast Universal PCR Mastermix (2X, Applied Biosystems AB Ref. 4352042), *Acanthamoeba* forward (0.5uM) and reverse primers (0.5uM), *Acanthamoeba* FAM-TAMRA probe (0.5uM), PhHV-forward (0.5uM) and reverse primers (0.5uM), PhHV-VIC-TAMRA probe (0.5uM) and 12.5 µl of the DNA eluted in distilled water. The amplification and detection were carried out with the ABI Prism<sup>®</sup> 7500 sequence detector and cycling program consisted in one cycle at 95°C for 20 sec and 45 cycles at 95°C for 3 sec and 30 sec at 60°C. Measurements were followed automatically and the Ct value for each sample was determined according to the fluorescence signal exceeding the background limit of 0.07. Each run contained negative controls and controls with quantified number of cysts. Linearity, sensitivity, reproducibility and detection limits were assessed for all the strains tested pure and diluted in distilled water before and after DNA extraction. Retesting with the same extracts were conducted with the SmartCyclerII<sup>®</sup> equipment in 50 two-step cycles of 10 sec at 95°, and 65 sec at 60°C after mixing 12.5 µl of extracted DNA with 12.5 µl 2X TaqMan Universal PCR Master Mix (MNL 430449 AB) containing the primers and probes each at 0.5 µM.

### **Confirmation of negativity (absence of *Acanthamoeba*) by real-time SYBR Green PCR**

SYBR Green, [2-[N-(3-dimethylaminopropyl)-N-propylamino]-4-[2, 3-dihydro-3-methyl-(benzo-1, 3-thiazol-2-yl)-methylidene]-1-phenyl-quinolinium] is an intercalating dye which fluoresces only when bound to double-stranded products generated by PCR. The f-d-real-t PCR is associated

to SYBR Green reactions carried out in a second tube after mixing 5µl of the DNA extract with 12.5 µl of the Fast Start Universal SYBR Green Master Mix (AB 04 913850001), 0.25µl of the forward and the reverse primers (final concentration of each 300 nanomol), 1 Unit of UNG (AB UNG Ref: 03539 806001) and 9.5µL of sterile distilled water. (1 cycle at 50°C for 20sec, 1 cycle at 95°C 20 sec and 45 amplification cycles at 95°C for 3 sec and at 60°C for 30 sec; positive threshold was fixed at 100. In every series of testing SYBR Green results were validated only if the DNA extracts from the reactants and the environment yielded negative results.

### **Specificity and interferences of *Bacteria* and fungi**

The potential interferences on the f-d-real-t PCR were studied by mixing before extraction 20 cysts of the strain T4 with 200µl of suspensions containing 10<sup>6</sup> CFU/ml of Bacteria or fungi or 10<sup>6</sup> PFU/ml of 3 different *Herpesviridae* isolated from corneal ulcers.

### **Comparison with other real-time PCR**

Aliquots from the DNA extracts containing the IC obtained from clinical samples were tested simultaneously according to the primers, probes and experimental conditions for real-time PCR described by the authors.[16-9]

### **Results**

The new f-d-real-t PCR detects and quantifies in one run *Acanthamoeba* and assesses simultaneously the DNA extraction yields and the PCR inhibitors. The detection capacities of the f-d-real-t PCR (dilution versus last positive signal) are identical using the ABI PRISM 7500® or the SmartCyclerII®, and both detected at least 0.1 cyst/µl (the equivalent of 1 cyst or less in the original specimen). However, the signals triggered using TaqMan probes for *A. astronyxis* were repeatedly inferior to those obtained for all the other strains (Table 1) with detection limits 1 log inferior (1 cyst/µl). Nevertheless, using the same set of primers and SYBR Green it was possible to detect at least 0.1 cyst/µl, suggesting a reduction in the annealing capacities of the of *A. astronyxis* probe targeted sequence. On the above, the f-d-real-t PCR associates systematically a SYBR Green PCR (5 µl of the DNA extract) before the absence of *Acanthamoeba* can be stated.

In Table 2 are presented the results obtained after mixing *Acanthamoeba* (20 cysts/sample) with highly concentrated bacterial, fungal or viral suspensions. The pathogens isolated from corneal infections did not alter the detection capacities of the f-d-real-t PCR. The Ct values obtained for the controls for each sample compared to the blanks were never delayed for more than 1.5 cycles indicating high and reproducible DNA recovery yields after the pre proteolysis.[22] All samples testing f-d-real-t PCR negative were SYBR Green negative and all negative controls from air and reactants were negative by both methods. For all the samples the dispersion of the Cts (intra and inter assays) for the targeted sequences and for the ICs was < 1.5 cycles. The SYBR Green PCR always detected 0.1 cyst/ $\mu$ l or less. During the preliminary SYBR Green validation procedures highly concentrated suspensions of *Candida* (*C. albicans*, *C. parapsilopsis*, *C. glabrata*, *C. krusei* and *Geotricum candidum* (but not the culture media) isolated from corneal lesions triggered weak signals that could have been misinterpreted. However, when the samples containing Bacteria, *Acanthamoeba* or fungi were tested after pre setting for the 2 equipments tested here the threshold value at 100, only the samples containing *Acanthamoeba* were positive.

The results obtained with 20 samples obtained from patients with signs evoking AK tested a-by classic routine diagnosis techniques (direct microscopic examination after Giemsa and Gram staining and culture for aerobic, anaerobic Bacteria, fungi and *Acanthamoeba*); b-by the real-time PCR [16-9] and c-by the new f-d-real-t PCR are presented in Table 3. The direct microscopic examination detected positive 60% of the samples and the culture of *Acanthamoeba* from corneal scrapings detected positive 7 out 10 samples (70 %). The potential PCR inhibitors were eliminated (reproducible for the ICs) and the isolates genotypically characterized belong to genotypes T 4 (70%), T 3; T5 and T 11 (10% each). Hundred % of the positive f-d-real-t PCR results were confirmed by SYBR Green. In 3 different experiments the dispersion of the Cts (intra and inter assays) for the targeted sequences and for the ICs were < 1.5 cycles for all the samples The real-time PCR using primers and probes bracketing other regions of the genome [16-9] detected as positive 8 out 10 samples, but the new f-d-real-t PCR detected 10 out 10, and all were confirmed by SYBR Green and genotypically characterized. Moreover, the false positive results obtained with the real-time PCR for samples containing *Salmonella lexinton*, *Clostridium sporulans*, *Serratia marcescens* and *Propionibacterium acnes* (Cts > 37) were repeatedly negative with the f-d-real-t PCR and confirmed as negative by SYBR Green

## Discussion



A new test was developed for the diagnosis of *Acanthamoeba*. The TaqMan probe selected does not cross react with prokaryote or eukaryote genome sequences, and as predicted by the comprehensive *in-silico* blast analysis the suspensions of *Acanthamoeba* mixed with other infectious agents do not produce false positive or negative results. To minimize misdiagnosis for samples containing low loads of *A. astronyxis* the SYBR Green procedure was systematically carried out. The SYBR Green may detect traces of residual double-stranded DNA and therefore, we limited its use for the confirmation of the absence of *Acanthamoeba*.

The culture methods may produce false negative results due to low parasitic load, small sample volumes and the use of antiseptic or antibiotics prior to sampling. Nevertheless, NAATs are independent of the growth of the microorganisms and may detect low amounts of injured *Acanthamoeba* even those suspended in antiseptics.[9, 11, 13-4, 23-4] Several PCRs and real-time PCRs have been described for diagnosis of *Acanthamoeba*, but none reported their abilities to detect the species available from the ATCC.[16-9,24] In addition, the sensitivity of real-time PCR were generally assessed testing the genotype T4 (the most frequently detected in corneal samples) and as shown in the present study these tests do not detect all the genotypes associated with AK. Moreover, the specificity of the f-d-real-t PCR is higher compared to the other real-t PCRs [17-9] especially for samples that contain one of the 4 bacteria that triggered positive signals. These false positive conclusions produced by the real-t PCR were confirmed by an additional *in-silico* testing showing partial homologies between the *Acanthamoeba* sequences previously selected for the real-time PCR [16-19] and the genomes of bacterial species (NCBI Blastn FAQs, ncbi.nlm.nih.gov.Genbank).

## Conclusion

The methods previously described for diagnosis of *Acanthamoeba* do not fulfill the expectancies for sensitive and specificity. None of them reported the capacity for detecting low levels of the ATCC strains of *Acanthamoeba* associated with pathology. The f-d-real-t PCR diagnosis strategy (f-d-real-t PCR coupled to SYBR Green) allows obtaining results within 2 h with minimal risk for false negative diagnosis for *A. astronyxis*. On 20 clinical samples the sensitivity and specificity of the f-d-real-t PCR is higher compared to those of the real-t PCR previous described.[16-9]

The on time interventions inpatients with AK improve the prognosis. Nevertheless, the therapeutic

management of AK is based on long lasting topical solutions administered repeatedly -at the beginning during day and night-, and most of the biocidal agents are toxic for the epithelium. On the above, false positive diagnosis may induce unnecessary iatrogenic toxic effects on the eye surface.[2, 4, 9-11, 24] In summary, the new real f-d-real-t PCR targeting sequences shared by all the *Acanthamoeba* available from the ATCC appears as a specific, sensitive, reproducible, quantitative and easy to perform tool. The diagnosis strategy can be adapted to the different real-time PCR platforms and produces results in less than 2 h with a significant low cost of reactants. To our knowledge, the f-d-real-t PCR is the first NAAT detecting and quantifying simultaneously all of the ATCC *Acanthamoeba* strains (including the genotype T5 and T11) that assesses simultaneously the DNA extraction yields and the presence of PCR inhibitors. Larger trials are necessary to confirm the usefulness of the f-d-real-t PCR for disease management and environmental studies.

**Word count: 2300**

**Table 1: Comparison of the detection capacities of the f-real time PCR**

0.1cyst/ $\mu$ l of the strain:	simplex <sup>a, c</sup>	duplex <sup>b, c</sup>
<i>A. comandoni</i>	+	+
<i>A. palestinensis</i> (ref 50708);	+	+
<i>A. sp ATCC ref 50655</i>	+	+
<i>A. griffini</i>	+	+
<i>A. palestinensis</i> (ref 30870);	+	+
<i>A. castellani</i>	+	+
<i>A. lenticulata</i>	+	+
<i>A. astronyxis</i>	+*	+*
<i>A. tubiashi</i>	+	+
<i>A. hatchetti</i>	+	+

The Cts for the internal controls were ranges for <sup>a</sup> between 29.8 and 31.3 and for <sup>b</sup> between 30.9 and 31.9. \*: The signals registered on the FAM channel were weaker than those obtained for the other strains and were triggered after  $37 \pm 2$  cycles of amplification. <sup>c</sup>: The real-time PCR using SYBR Green confirmed positive results (peak at 78°C); simplex: f-real time PCR for *Acanthamoeba* and for IC in separate tubes; duplex: both tests in the same tube; +: positive; -:negative;

**Table 2: Analysis of the potential interferences of *Bacteria*, fungi and viruses on the f-d-real-t PCR**

microorganisms introduced in the tubes before DNA extraction	Ct for <i>Acanthamoeba</i> (FAM)	Ct for the Internal control (VIC)	Conclusions
Acanthamoeba + PBS	30	33.0	P
Acanthamoeba+ <i>Staphylococcus capitis</i>	30.3	33.0	P
PBS + <i>Staphylococcus capitis</i>	>45	32.4	N
Acanthamoeba + <i>Staphylococcus aureus</i>	30.6	33.0	P
PBS + <i>Staphylococcus aureus</i>	>45	32.0	N
Acanthamoeba + <i>Streptococcus pneumoniae</i>	29.9	32.9	P
PBS + <i>Streptococcus pneumoniae</i>	>45	31.9	N
Acanthamoeba + <i>Strepto. B-haemolyticus</i>	30.1	32.9	P
PBS + <i>Strepto. B-haemolyticus</i>	>45	33.9	N
Acanthamoeba + <i>Propionibacterium acnes</i>	29.9	32.9	P
PBS + <i>Propionibacterium acnes</i>	>45	33.4	N
Acanthamoeba + <i>Serratia marcescens</i>	30.4	32.9	P
PBS + <i>Serratia marcescens</i>	>45	31.9	N
Acanthamoeba + <i>Acinetobacter freundii</i>	30.2	32.8	P
PBS + 1000 <i>Acinetobacter freundii</i>	>45	32.1	N
Acanthamoeba + <i>Stenotrophomonas maltophilia</i>	29.6	33.1	P
PBS + <i>Stenotrophomonas maltophilia</i>	>45	32.4	N
Acanthamoeba + <i>Pseudomonas aeruginosa</i>	30.3	31.9	P
PBS + <i>Pseudomonas aeruginosa</i>	>45	31.8	N
Acanthamoeba + <i>Corynebacterium sp.</i>	30.5	33.0	P
PBS + <i>Corynebacterium sp.</i>	>45	32.9	N
Acanthamoeba + <i>Escherichia coli</i>	30.6	33.1	P
PBS + <i>Escherichia coli</i>	>45	32.2	N
Acanthamoeba + <i>Candida albicans</i>	30.8	33.2	P
PBS + <i>Candida albicans</i>	>45	31.9	N
Acanthamoeba + of <i>Candida glabrata</i>	30.8	33.1	P
PBS + <i>Candida glabrata</i>	>45	32.2	N
Acanthamoeba + <i>Candida krusei</i>	30.2	33	P
PBS + <i>Candida krusei</i>	>45	32.4	N
Acanthamoeba + <i>Candida lusitaniae</i>	30.2	32	P
PBS + <i>Candida lusitaniae</i>	>45	31.9	N
Acanthamoeba + <i>Candida parapsilopsis</i>	30	32	P
PBS + f <i>Candida parapsilopsis</i>	>45	32	N
Acanthamoeba + <i>Aspergillus sp.</i>	30	33.1	P
PBS + <i>Aspergillus sp.</i>	>45	31.9	N
Acanthamoeba + <i>Fusarium sp.</i>	30.2	33.2	P
PBS + <i>Fusarium sp.</i>	>45	32.5	N
Acanthamoeba + <i>Scedosporium sp.</i>	30	33.1	P
PBS + <i>Scedosporium sp.</i>	>45	32.0	N
Acanthamoeba + <i>Curvularia sp.</i>	30.3	32.9	P
PBS + <i>Curvularia sp.</i>	>45	32.1	N
Acanthamoeba + <i>Phoma sp.</i>	30.1	33	P
PBS + <i>Phoma sp.</i>	>45	32	N
Acanthamoeba + <i>Acremonium sp.</i>	31.0	33.1	P
PBS + <i>Acremonium sp.</i>	>45	32	N
Acanthamoeba + <i>Herpes simplex virus type 1**</i>	30.3	33.0	P
PBS + <i>Herpes simplex virus type 1**</i>	>45	33	N
Acanthamoeba + <i>Herpes simplex virus type 2**</i>	30.5	32.2	P
PBS + <i>Herpes simplex virus type 2**</i>	>45	31.1	N
Acanthamoeba + <i>Varicella zoster virus**</i>	30.4	32.8	P
PBS + <i>Varicella zoster virus**</i>	>45	32.1	N

°: 20 cysts of *Acanthamoeba* were mixed with other microorganisms ( $10^6$  CFU/ml of *Bacteria* or fungi or  $10^6$  PFU/ml of \*\*viruses). P: Positive results, confirmed by SYBR Green. N: f-d-real t PCR negative confirmed by SYBR Green

**Table 3: Performances of f-d-real-t PCR compared to other techniques on 20 samples obtained from subjects presenting with signs evoking AK**

microscopic examination Cysts (Yes/No)	culture	real-t PCR [16-9] #	f- duplex real-t PCR #	SYBR Green	Genotype
Y	P A	P	P**	P	T4
Y	N A	P	P**	P	T11
N	N A	P	P**	P	T3
N	P A	N##	P**	P	T5
Y	P A	P	P**	P	T4
Y	N A	N##	P**	P	T4
N	P A	P	P**	P	T4
Y	P A	P	P**	P	T4
N	P A	P	P**	P	T4
Y	P A	P	P**	P	T4
N	<i>P. aeruginosa</i>	N	N	N	
N	<i>S. aureus</i>	N	N	N	
N	<i>Salmonella lexinton</i>	P*	N	N	
N	<i>S. pyogenes</i>	N	N	N	
N	<i>P. aeruginosa</i>	N	N	N	
N	<i>A. freundii</i>	N	N	N	
N	<i>Clostridium sporulans</i>	P*	N	N	
N	<i>Serratia marcescens</i>	P*	N	N	
N	<i>P. acnes</i>	P*	N	N	
N	<i>E. coli</i>	N	N	N	

P A: Positive culture for *Acanthamoeba*; N A: Negative culture for *Acanthamoeba*; P: Positive real-time PCR; N: Negative real-time PCR; \*: Ct>35; #: No inhibition for the IC (Cts ranged between 31.5 and 33); ##: Ct> 42; \*\*: Ct<38

## References

- [1] Mergeryan H. The prevalence of *Acanthamoeba* in the human environment. *Rev. Infect. Dis.* 1991;13 (Suppl. 5):S390–S391
- [2] Marciano-Cabral F. *Acanthamoeba* spp. as Agents of Disease in Humans. *Clin. Microbiol. Rev.* 2003; 16(2):273–307
- [3] Bhagwande SB, Carter RF, Naik KG et al. A case of hartmannellid amebic meningoencephalitis in Zambia. *Am. J. Clin. Pathol.* 1975; 63:483–492
- [4] Petry F, Torzewski M, Bohl J, Wilhelm-Schwenkmezger T, Scheid P, Walochnik J, Michel R, Zöller L, Werhahn KJ, Bhakdi S, Lackner KJ. Early diagnosis of *Acanthamoeba* infection during routine cytological examination of cerebrospinal fluid. *J Clin Microbiol.* 2006; 44:1903-4
- [5] Illingworth CD, Cook SD. *Acanthamoeba* keratitis. *Surv Ophthalmol* 1998; 42:493-508
- [6] Walochnik J, Haller-Schober E, Kolli H et al. Discrimination between clinically relevant and nonrelevant *Acanthamoeba* strains isolated from contact lens- wearing keratitis patients in Austria. *J. Clin. Microbiol.* 2000; 8:3932–3936
- [7] Clarke DW, Niederkorn JY. The pathophysiology of *Acanthamoeba* keratitis. *Trends Parasitol* 2006; 22:175-80

[8] Sharma S, Pasricha G, Das D, Aggarwal RK. *Acanthamoeba* keratitis in non-contact lens wearers in India: DNA typing-based validation and a simple detection assay. *Arch Ophthalmol* 2004; 122:1430-4

[9] Schaumberg DA, Snow KK, Dana MR. The epidemic of *Acanthamoeba* keratitis: where do we stand? *Cornea*. 1998;17:3-10

[10] Cardine S, Bourcier T, Chaumeil C, Zamfir O, Borderie V, Laroche L. Prise en charge clinique et pronostic des k ratites amibiennes. Etude r trospective   propos de 25 cas. *J fr Ophthalmol* 2002;25 :1007-1013

[11] Mathers WD. *Acanthamoeba* : a difficult pathogen to evaluate and treat. *Cornea* 2004; 23:325

[12] Lehmann OJ, Green SM, Morlet N, Kilvington S, Keys MF, Matheson MM, Dart JK, McGill JI, Watt PJ. Polymerase chain reaction analysis of corneal epithelial and tear samples in the diagnosis of *Acanthamoeba* keratitis. *Invest Ophthalmol Vis Sci* 1998; 39:1261-5

[13] Van Gelder RN. Applications of the polymerase chain reaction to diagnosis of ophthalmic disease. *Surv Ophthalmol*. 2001;46(3):248-58

[14] Yagi S, Booton GC, Visvesvara GS, Schuster FL. Detection of *Balamuthia mitochondrial* 16S rRNA gene DNA in clinical specimens by PCR. *J Clin Microbiol* 2005; 43:3192-7

[15] Schroeder JM, Booton GC, Hay J, Niszl IA, Seal DV, Markus MB, Fuerst PA, Byers TJ. 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

[16] Qvarnstrom Y, Visvesvara GS, Sriram R, da Silva AJ. Multiplex real-time PCR assay for the simultaneous detection of *Acanthamoeba* spp., *Balamuthia mandrillaris*, and *Naegleria fowleri*. *J Clin Microbiology* 2006;44:3589-95

[17] Rivière D, Ménard Szczebara F, Berjeaud JM, Frère J, Héchard Y. Development of a real-time PCR assay for quantification of *Acanthamoeba* trophozoites and cysts. *J Microbiol Methods* 2006;64:78-83

[18] Zamfir O, Yera H, Bourcier H, Batellier L, Dupouy-Camet J, Tourte-Schaeffer C, Chaumeil C. Diagnostic par PCR des kératites à *Acanthamoeba* spp. *J. Fr. Ophthalmol.* 2006;29:1034-40

[19] Yera H, Zamfir O, Bourcier T, Ancelle T, Batellier L, Dupouy-Camet J, Chaumeil C. Comparaison of PCR, microscopic examination and culture in the early diagnosis and characterization of *Acanthamoeba* isolates from ocular infections. *Eur. J. Clin. Microbiol. & Inf. Dis.* 2007;26:221-4

[20] Goldschmidt P, Rostane H, Saint-Jean C, Batellier L, Alouch C, Zito E, Bourcier T, Laroche L, Chaumeil. 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(11):1354-6

[21] Van Doornum G, Guldemeester J, Osterhaus ADME et al. Diagnosing herpesvirus infections by real-time amplification and rapid culture. *J of Clin Microbiol.* 2003;41:2576-580



[22] Goldschmidt P, Degorge S, Saint-Jean C, Yera H, Zekhnini F, Batellier L, Laroche L, Chaumeil C. Resistance of *Acanthamoeba* to classic DNA extraction methods used for the diagnosis of corneal infections. *Br J Ophthalmol* 2008;92:112-5

[23] Epstein RJ, Wilson LA, Visvesvara GS, Plourde EG Jr. Rapid diagnosis of *Acanthamoeba* keratitis from corneal scrapings using indirect fluorescent antibody staining. *Arch Ophthalmol* 1986;104:1318-21

[24] Behets J, Declerck P, Delaedt Y, Verelst L, Ollevier F. Quantitative detection and differentiation of free-living amoeba species using SYBR green-based real-time PCR melting curve analysis. *Curr Microbiol.* 2006;53(6):506-9