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Resistance of *Acanthamoeba* to classic DNA extraction methods used for the diagnosis of corneal infections

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ABSTRACT

Aims: Sensitive diagnosis of *Acanthamoeba* infections may prevent the clinical condition from becoming worse. In order to improve the diagnosis tool performances, we studied the implication of the DNA extraction procedures on the detection of *Acanthamoeba* by real-time PCR.

Methods: *Acanthamoeba* cysts mixed with a tag virus were processed according to different DNA preparation procedures: heat, Proteinase K (ProtK), alkali lysis, QIAamp kit®, MagNA Pure (DNA Mini kit, MagNA Pure® Nucleic Acid isolation kit), ProtK+QIAamp and ProtK+MagNA Pure. Parasite-DNA loads were assessed by real-time PCR.

Results: The results show that the structures of *Acanthamoeba* cysts are resistant to reagents releasing the DNA from other cells and viruses. Heat, NaOH or ProtK did not allow the DNA extraction yields to be assessed or the inhibitors to be eliminated. The QIAamp and the MagNA Pure partially improved the sensitivity of the PCR and eliminated the inhibitors. A significant increase in positive results was obtained with a ProtK treatment before commercial extraction kits.

ProtK+MagNA Pure yielded the highest rates of positivity.

Conclusion: To minimise false negative results, the nucleic-acid based *Acanthamoeba* diagnosis requires, first, the efficient lysis of cysts (without affecting the DNA) to make the DNA available for extraction and amplification, and, second, the elimination of PCR inhibitors. A significant increase in the detection rates is obtained by adding a ProtK treatment (10 min at 56°C) before the commercial procedures. ProtK+MagNA Pure yielded the best results in 30 min, followed by ProtK+QIAamp (150 min).

Acanthamoeba are free-living organisms with a life cycle characterised by two stages, an actively dividing trophozoite developing hyaline pseudopodia and a quiescent cyst. *Acanthamoeba* are present in air, soil, dust, air-conditioning units, treated water, bottled water, swimming pools, dialysis units, eyewash stations, contact lenses and lens cases, and are considered among the most prevalent eukaryotic protozoa found in the environment.¹⁻⁸

The trophozoites contain a trilaminar plasma membrane with spiny surface projections and a contractile vacuole in the cytoplasm, and their replication occurs by binary fission.⁸⁻⁹ Cyst formation is triggered under adverse conditions such as changes in temperature, pH and desiccation.¹⁰⁻¹⁵

The analysis of full-length nuclear small-subunit (SSU) rRNA gene allows *Acanthamoeba* to be classified into 15 genotypes (T1 to T15).¹⁶⁻¹⁸

Acanthamoeba can provoke sinusitis, cutaneous lesions, meningoencephalitis and keratitis in humans,¹⁹⁻²³ and the majority of types causing keratitis belong to sequence T4.²⁴⁻²⁷

The nucleic acid amplification techniques (NAATs) generated a breakthrough in the history of parasitic infestation diagnosis, enhancing dramatically the sensitivity of the diagnosis methods and reducing the time needed by a lab to produce results.²⁸⁻²⁹ The samples (biopsies, body fluids, tissue scraping and cells) should be processed (extraction) before NAATs in order to eliminate all the material (proteins, lipids, polysaccharides, anticoagulants, etc.) that may inhibit the DNA polymerases. The extraction of DNA consists in nucleic acid isolation, purification and concentration in an eluted product, and several methods have been adapted to viral, fungal, bacterial, chlamydial and parasitic PCR diagnosis.²⁸⁻³²

While evaluating the performances of different NAATs for corneal *Acanthamoeba* keratitis diagnosis, we observed significant differences if the results of the PCR calibration curves (Ct vs number of cysts) were plotted with suspensions of *Acanthamoeba* diluted before DNA extraction compared with those obtained with samples in which the DNA was extracted from a concentrated suspension and diluted thereafter. To understand these discrepancies, we planned a series of experiments aimed to study specifically for *Acanthamoeba* the efficiency of the different DNA extraction procedures routinely used in clinical microbiology settings.

MATERIAL AND METHODS

Acanthamoeba preparations and nucleic acid extraction and purification

All the experiments were carried out using the reference strain of *Acanthamoeba* T4 purchased from the American Type Cell Collection (ATCC). Trophozoites were cultivated in flasks containing NaCl 0.9% and killed *E. coli*. The growth was assessed by direct microscopic examination.

In order to induce the total transformation of the trophozoites into cysts, the suspensions were kept at 4°C for 48 h. A cyst count was performed using the HMX Beckman haematology cell counter, double-checked by a microscopic count in a Malassez cell and diluted. DNA was extracted from suspensions containing 10⁵, 10³, 30, 10 and 3 cysts/ml.



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DNA extraction

Five microlitres of a whole virus preparation of Seal Herpesvirus (phHV) (gift from van Doornum, Department of Virology Erasmus MC, Rotterdam, The Netherlands) was added to 200 µl of each cyst suspension before extraction (final concentration of approximately 1000–2000 viral particles per ml) to monitor the extraction processes and to assess the potential PCR inhibitors through the detection of delays in specific signals.²⁹

Heat treatment

Dilutions of the cyst suspensions (200 µl) were mixed with 5 µl of phHV and heated in a dry platform for 10 min at 95°C, placed for 5 min at –20°C and kept for 30 min at 20°C. PCR was carried out immediately after.

Proteinase K (ProtK) treatment

Different concentrations of cysts suspended in 200 µl of PBS were mixed with 200 µl of ATL buffer (Qiagen, Courtaboeuf, France)+40 µl ProtK (Qiagen)+5 µl of phHV. The tubes were incubated at 56°C for 10, 60 or 240 min. The enzyme was inactivated at 95°C for 10 min, and the tubes were cooled (–20°C) for 5 min. The temperature was then stabilised for 30 min at 20°C, and PCR was carried out immediately thereafter. For further DNA extraction (QIAmp or MagNA Pure), 200 µl of the suspensions treated with ProtK was processed immediately afterwards, according to the QIAmp or the MagNA Pure kit instructions, and eluted in 50 µl of DNA-free distilled water.

NaOH

Different concentrations of cysts were resuspended in 100 µl of NaOH 0.1 M+5 µl of phHV and incubated at 95°C for 5 min. PBS (200 µl) was added, and the tubes were cooled (–20°C) for 5 min. The temperature was stabilised for 30 min at 20°C before PCR or before the additional extraction procedures carried out with 200 µl of the treated suspensions, according to the instructions provided by the QIAmp or the MagNA Pure manufacturers, and eluted in 50 µl of DNA-free distilled water.

QIAmp and MagNA Pure

QIAmp® DNA Mini kit and MagNA Pure® Nucleic Acid isolation kit
Different concentrations of cysts were resuspended in 200 µl PBS+5 µl of phHV, and the DNA extraction was performed manually by the solid column-based extraction kit QIAmp® DNA Mini kit (tissue protocol) or by the MagNA Pure® Nucleic Acid isolation kit (Roche, Basle, Switzerland) with the MagNA Pure® Compact (Roche) automate. Nucleic acids were eluted in 50 µl of DNA free distilled water. The time needed for the manual QIAmp procedure was 120 min with no additional equipment required and for MagNA Pure less than 28 min, but required a specific dedicated robot.

Primers and probe

The sequences for *Acanthamoeba* real-time PCR were selected by using the primer Express 1.0 software® Applied Biosystems (Courtaboeuf, France) and blasted using the NCBI Blastn FAQs®. These primers bracket a highly conserved sequence coding for the mitochondrial SSU rRNA in the vast majority of strains of *Acanthamoeba*.²⁷

Real-time TaqMan® PCR assays

Two PCR reactions were carried out for each test in different microtubes in a final volume of 25 µl. The first tube used for the detection of *Acanthamoeba* DNA included the forward primer (0.5 µM), the reverse primer (0.5 µM) and the FAM-TAMRA *Acanthamoeba* probe (0.4 µM) in 2× TaqMan® Universal PCR Master Mix (MNL 430449, Applied Biosystems) and 12.5 µl of the isolated DNA eluted in distilled water. The second tube included the phHV forward primer (0.5 µM), reverse primer (0.5 µM), the VIC-TAMRA phHV probe (0.4 µM) and 12.5 µl of the isolated DNA eluted in distilled water.

After incubation for 2 min at 50°C with uracil *N*'-glycosylase, the microtubes were incubated for 10 min at 95°C. The PCR cycling programme consisted in 50 two-step cycles of 10 s at 95°, and 65 s at 60°C. The amplification and detection were carried out using the ABI Prism 7500 sequence detector system (Applied Biosystems). Real-time measurements were automatically registered, and the Ct were determined according to the fluorescence signal exceeding the background limit of 0.20. The results (corresponding to the number of cysts) were corrected according to the volume of reactants used for the different extraction procedures.

RESULTS

The results presented in table 1 show that heating the samples for 10 min at 95°C or the treatment only with NaOH did not significantly enhance the *Acanthamoeba* detection levels. Treatment of samples for 10 min with 0.1 M NaOH did hydrolyse the phHV DNA, thus limiting the assessment of the nucleic acid extraction yields and of the inhibitors.

The treatment with ProtK slightly improved *Acanthamoeba* detection, but notably, no direct impact on the release of DNA was observed after prolonging the proteolysis for more than 10 min at 56°C. In previous experiments, the overnight proteolysis of cysts at 4°C, 20°C and 37°C did not improve the detection rates (results not shown). In the present study, the treatment with ProtK may have slightly improved the detection of *Acanthamoeba* but was unable to eliminate the inhibitors. The proteolytic treatment for 4 h at 56°C did not eliminate the inhibitors in any of the samples, thus suggesting that the nature of the inhibitors was different from that of polypeptide-related structures.

The QIAmp and the MagNA Pure carried out according to the routine procedures were able to improve partially the sensitivity of the real-time PCR with almost equivalent performances. Both kits did eliminate the inhibitors from all the proteolytic mixtures (no delays in phHV Cts were observed), and it could be concluded that no parasites were in the samples testing negative. However, more than a threefold increase in the detection rates was obtained (with no inhibition in any of the reactions) if the samples were treated for 10 min with ProtK before carrying out the commercial DNA-extraction procedures. The cost of reactants was equivalent for both techniques, but the time required for ProtK+QIAmp and ProtK+MagNA Pure was 160 min (manual) and 30 min (robot), respectively. Positive results for suspensions containing very low cyst concentrations (3 cysts/ml corresponding to 0.15 cysts/reaction tube) were obtained in one test out of three only for samples pretreated with ProtK before MagNA Pure.

DISCUSSION

Acanthamoeba cysts are resistant to normal chlorination, extreme temperatures, most biocides and antibiotics, but not

Table 1 Detection of *Acanthamoeba* by real-time PCR (results are indicated as + (positive) or – (negative) for each series of testing)

Treatment of the specimen before DNA amplification	No. of cysts per ml of suspension (before DNA extraction)				
	1000 (I)	100 (II)	30 (III)	10 (IV)	3 (V)
None	+++	+ – –	– – –	– – –	– – –
95 °C for 10 min*	+++	+ – –	– – –	– – –	– – –
NaOH 0.1 M†	+++	+ – –	– – –	– – –	– – –
ProtK for 10 min‡	+++	+ – –	+ – –	– – –	– – –
ProtK for 60 min‡	+++	+ – –	+ – –	– – –	– – –
ProtK for 240 min‡	+++	+ – –	+ – –	– – –	– – –
QIAmp® manual§	+++	+++	+++¶	–¶ –¶ –¶	–¶ –¶ –¶
ProtK for 10 min+QIAmp‡	+++	+++	+++	+ –¶ –¶	–¶ –¶ –¶
ProtK for 60 min+QIAmp‡	+++	+++	+++	+ –¶ –¶	–¶ –¶ –¶
ProtK for 240 min+QIAmp‡	+++	+++	+++	+ –¶ –¶	–¶ –¶ –¶
NaOH 0.1 M†+ QIAmp	+++	+++	+++	+ – –	– – –
MagNA Pure®**	+++	+++	+++	+ –¶ –¶	–¶ –¶ –¶
ProtK for 10 min+MagNA Pure‡	+++	+++	+++	+++	+ –¶ –¶
ProtK for 60 min+MagNA Pure‡	+++	+++	+++	+++	+ –¶ –¶
ProtK for 240 min+MagNA Pure‡	+++	+++	+++	+ –¶ +	–¶ –¶ –¶
NaOH 0.1 M†+MagNA Pure	+++	+++	+ – –	+ – –	– – –

(I) to (V): concentrations corresponding to 50, 5, 1.5, 0.5 and 0.15 cysts/PCR reaction tube.

*Specimens in microtubes were placed in a dry heater for 10 min at 95 °C and kept at 20 °C for 30 min before amplification; †samples were treated with NaOH (0.1 M final concentration) at 95 °C for 5 min; ‡ProtK at 56 °C and heat inactivation at 95 °C; §QIAmp® DNA Mini Kit (Qiagen)—manual method; ¶true negative values (no delays were observed for the pHV Cts while comparing each result to those obtained with the controls for pHV, indicating the absence of inhibitors; samples showing delays or more than two Cts in comparison with controls were considered inhibited); **MagNA Pure® (Roche)—automatic extraction robot.

to methylene oxide or autoclaving.^{33–37} The mature cysts are surrounded by two thick walls which form the exocyst and an endocyst that is closely apposed to the cell membrane. The nuclear membrane forms projections with vesicles bounded by double membranes, and very dense bodies of different sizes are found in the nucleus of every cyst.^{38–39} This high resistance of the cyst walls and the nuclear proteins that keep DNA condensed may explain the results that could have been obtained by using DNA-extraction procedures validated for viruses or bacteria if cysts are not properly lysed; it should be expected that the annealing of the primers and probes cannot be achieved because the parasite DNA is not available.

For micro-organisms different from cysts, several authors have shown the direct implication of DNA extraction on the performance of NAATs. Four DNA techniques, 65 °C phenol, incubation at 97 °C, ProtK and QIAmp tissue kit, tested for the diagnosis of intracellular bacteria (*Chlamydia trachomatis*) showed that the QIAmp and the hot phenol extraction allowed the highest detection and that the digestion with ProtK and the heat denaturation were unable to eliminate inhibitors.⁴⁰ For other bacteria, six methods (QIAmp Blood kit, Roche high PCR template, Puregene, boiling, glass beads/sonication and wash/alkali/heat lysis) were evaluated in blood culture bottles infected with Gram-negative and -positive bacteria, yeast and fungi. The wash/alkali/heat lysis method was the most sensitive, reproducible and cost-effective method and removed the PCR inhibitors.⁴¹ For the diagnosis of *Candida*, *Aspergillus* and *Cryptococcus* sp., the extraction of DNA carried out using either the inhouse guanidium isothiocyanate-silica method⁴² or the QIAmp tissue kit yielded the same results if, before extraction, a minibeat beating treatment was carried out to mechanically disrupt the yeasts.⁴³ These results were confirmed by another study showing, for *Candida albicans* and *Aspergillus niger*, that QIAmp yielded the same sensitivity as one inhouse technique but shortened the duration of DNA extraction.⁴⁴ For non-specific ophthalmic pathogens, a study carried out with *Pneumocystis carinii* sp. *hominis* mixed with human blood comparing the performances of three kits (GeneReleaser,

QIAmp, ReadyAmp, ProtK, and ProtK-phenol-chloroform treatment) showed that QIAmp was the most effective.⁴⁵ For plasmodium, rapid boiling and the STIX provided the best results overall.⁴⁶

All the trials described previously compared the different procedures according to the number of positive PCR results, but no systematic assessment of the DNA yields extracted from the samples was reported. To our knowledge, this is the first report showing—with the inclusion of an internal control (pHV serving a tag)—that the highly resistant structure of cysts may explain that the inability of the reagents used to extract the DNA from *Acanthamoeba* cysts leads to a false negative diagnosis. In the present study, the semiquantification of *Acanthamoeba* was determined simultaneously in the same tube with the DNA extraction yields and with the assessment of PCR inhibitors. Neither heating the cyst suspensions nor the ProtK treatment nor the commercial manual or automatic methods used separately produced satisfactory results. However, treatment with ProtK for 10 min before QIAmp or MagNA Pure, as used routinely for corneal infection diagnosis,^{28–32} dramatically improved the sensitivity of *Acanthamoeba* detection (10 and 3 cysts/ml, respectively).

The low performances of the *Acanthamoeba* diagnosis methods could be suspected to be due to a reduced avidity of the solid supports included in the commercial kits to bind the DNA and to the inefficiency of the DNA-elution procedures indicated by the manufacturers. Nevertheless, none of these hypotheses could be confirmed because the signals produced by the pHV were identical to the respective controls. Moreover, for all the samples extracted by QIAmp or MagNA Pure (but not for samples treated with NaOH or with ProtK), the pHV Cts were identical to each of the respective controls (pHV diluted in PBS and extracted), thus indicating that the negative results for *Acanthamoeba* were not the result of intrinsically low DNA-extraction capacities of the kits but signify that the DNA was not released from the cysts. After processing samples using the QIAmp or the MagNA Pure DNA extraction procedures, the Cts obtained for pHV with the samples testing negative for

Acanthamoeba showed no inhibition. Finally, the addition of a 10-min Prot K pretreatment to the commercial procedures generated a higher number of positive results: the *Acanthamoeba* cyst DNA was prepared for further extraction processes, and the Taq polymerase inhibitors were eliminated. These two goals were achieved without affecting the assessment of the DNA-extraction yields.

For the detection of *Acanthamoeba* the material representing the site where they may replicate and/or persist has to be adequately processed in the laboratory, and the diagnosis of this parasitic keratitis remains one of the most challenging issues in microbiology, due first to the extremely reduced biological material that can be obtained from patients presenting with small corneal lesions. Because the intense and repeated scraping of the cornea may trigger inflammatory responses with unpredictable consequences for the transparent structures of the eye surface, the laboratory cannot count with systematic double-checking by asking the ophthalmologists to carry out additional samplings. To minimise the risks of false negative results that may reduce the benefits of appropriate treatments, it should be noted that DNA-extraction procedures are not efficient in eliminating fluorescein, rose Bengal, lissamine green and local anaesthetics known to inhibit the Taq polymerase (PCR inhibitors): corneal specimens should be collected after properly rinsing the eye surface.^{47, 48} In addition, as shown in the present study, inappropriate procedures before conducting NAATs can lead to a false diagnosis of this infestation.

In conclusion, for the diagnosis of *Acanthamoeba* infestation by NAATs (real-time PCR or other), the evaluation of the different DNA extraction strategies shows that the highest detection rates (10 cysts/ml) could be obtained by treating samples containing cysts with ProtK before DNA is extracted by the classic methods (QIAmp DNA manual kit or MagNA Pure robot).

Competing interests: None.

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LETTER

A simple technique to administer mydrinicaine in needle-phobic patients

Subconjunctival mydrinicaine injections are used in routine practice in patients with uveitis to prevent the formation of permanent posterior synechiae.¹ Mydrinicaine No 2 (Moorfields Eye Hospital formulation) contains a combination of 6 mg procaine hydrochloride, 1 mg atropine sulphate and 0.12 ml epinephrine solution (1 in 1000). As it is normally injected subconjunctivally, this may prove difficult to administer to anxious, young patients, especially those who are needle-phobic. Belonephobia, the abnormal fear of sharply pointed objects, especially needles, affects up to 10% of the population and has implications for treatment and follow-up.²

We describe a very simple but effective method of administering mydrinicaine in needle-phobic, uveitic patients. We use two small sterile cotton wool pledgets which are fully soaked in 0.3 ml mydrinicaine No 2 (which is usually used in adults <75 years of age). The patient receives topical anaesthetic drops into the eye. The lower lid is pulled downwards gently, and the first mydrinicaine-soaked pledget is placed securely with forceps into the inferior conjunctival fornix as the patient looks upwards (fig 1A). The second is tucked into the superior fornix under the upper eyelid as the patient looks downwards (fig 1B). Care should be taken to avoid abrading the cornea. The patient should be instructed to keep their eyes closed for 20–30 min. The pledgets can then be removed carefully. Topical steroid and mydriatic drops can subsequently be prescribed. The patient may be reviewed the following day to check the efficacy of the treatment. We have even successfully used this technique in a young Afro-Caribbean

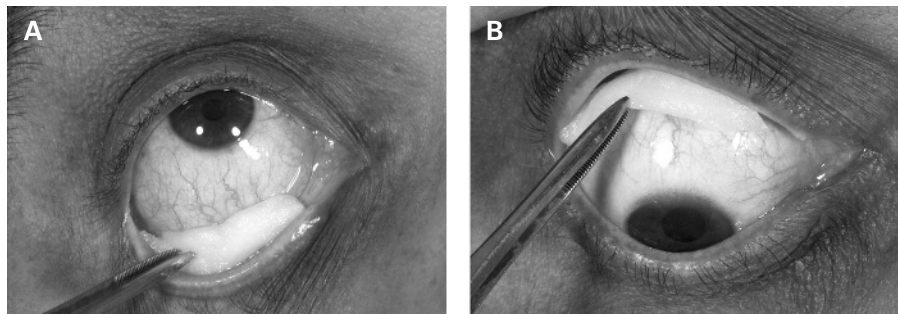


Figure 1 Mydrinicaine-soaked pledget placed with forceps into (A) the inferior conjunctival fornix and (B) the superior conjunctival fornix.

patient with black irides and severe fibrinous uveitis.

We propose using this pain-free technique in the outpatient setting for needle-phobic patients who require subconjunctival administration of a drug, to avoid the unnecessary use of local anaesthesia (with sedation) or general anaesthesia or the utilisation of valuable theatre time. It also avoids the complications of subconjunctival haemorrhage, chemosis and globe perforation.^{3,4} It may be worthwhile considering this technique in cooperative children (using mydrinicaine No 1) in order to avoid a general anaesthetic. We certainly advocate its use in the first instance, before considering further protracted interventions, as it is safe and effective. Indeed, this technique is a useful alternative to a subconjunctival injection for any patient, although the exact amount of drug absorbed through the conjunctiva is difficult to quantify, and also, it does not allow distribution of the drug in all quadrants, which would be useful in cases of 360° of posterior synechiae. This would therefore be a limitation of the technique in needle-phobic patients with 360° of posterior synechiae, in whom it may not be as effective as subconjunctival injections in breaking the synechiae.

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CORRECTION

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In the paper by Goldschmidt *et al* (*Br J Ophthalmol* 2008;**92**:112–15) the fourth author should be H Yera.