Real-time PCR for laboratory diagnosis of *Acanthamoeba* keratitis




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***Acanthamoeba* keratitis is a painful vision-threatening disease of the human cornea. It is characterised by severe ocular pain or partial paracentral stromal ring infiltrate, which can be frequently misdiagnosed as herpes simplex virus keratitis. If the infection is not treated promptly, it may progress to ulceration of the cornea, loss of visual acuity, possibly blindness and even require enucleation. *Acanthamoeba* sp are found commonly in freshwater, tap water, seawater, hot springs and swimming pools. An epidemiologic case study revealed that major risk factors were the use of contact lenses, predominantly extended-wear soft lenses, the use of homemade rinsing saline[1] and users who wear their lenses while swimming[1]. The conventional method of detecting the formation of oocysts of *Acanthamoeba* by a culture technique takes an average three–five days. DNA amplification by PCR can improve turnaround time for the diagnosis[2]. A study was carried out in this laboratory to compare the traditional culture method with a real-time PCR assay.**

Methods

Samples tested. During the period of June 2007–July 2010, *Acanthamoeba* culture and real-time PCR were performed on 285 samples from a total of 189 patients. Samples included corneal scrapings, corneal swab, anterior chamber tap and contact lens case solutions. Samples were transported from the eye clinics at Sydney Eye Hospital and The Prince of Wales Hospital by courier within the working day. Some patients were tested sequentially at intervals of one week.

***Acanthamoeba* culture technique.** An air-dried non nutrient water agar plate (Oxoid purified agar 18g/L) was flooded with a suspension of *E. coli* (10^7–10^8 cfu/ml) then drained and dried for 15 minutes. Specimens were inoculated directly onto the plate in a small area. The plate was examined after three, five and seven days for the presence of *Acanthamoeba* cysts using phase contrast microscopy at 40x magnification. Negative cultures were reported after seven days, although incubation was pursued for a total of 14 days.

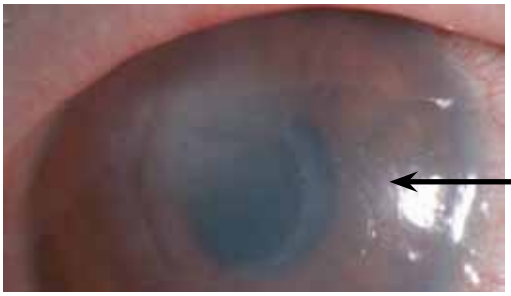
DNA extraction. Total DNA was extracted from specimens by the use of a semiautomatic robotic machine (MagNaPure, Roche, Australia). DNA extracts were stored at –20ºC before testing.

Real-time PCR assay. This PCR assay was based on a TaqMan-probe using Roche Fast Start DNA master mix hybridisation probes and a Light Cycler 2.0 real-time PCR machine[3]. This assay targets the 18S rRNA gene and utilises two primers and one TaqMan probe.

Forward primer 5' CCCAGATCGTTTACCGTGAA-3',

Reverse primer 5'-TAAATATTAATGCCCCCAACTATCC-3' and

TaqMan probe 5'-FAM-CTGCCACCGAATACATTAGCATGG-BHQ1-3'[3] Each 20 µl PCR mixture contained 2 µl of FastStart DNA master hybridisation probes mix, 0.6 µl of forward and reverse primers, 0.2 µl of probe, 1.3 µl 25 mM magnesium, 10.3 µl nuclease free water and 5 µl of extract. Primers and probes were all used at a 20 µM working concentration. PCRs were performed in a Roche Light Cycler 2.0 machine with activation at 95ºC for 10 min, amplification at 95ºC for 10 sec, 60ºC for 30 sec (with single acquisition) for 50 cycles, followed by a final cooling step of 40ºC for 30 sec. The fluorescence signal above the threshold level was measured at the end of the annealing and extension plateau. Results were analysed using the LC4.0 software. The cycling took 60 minutes to complete. The PCR product could also



Stromal ring infiltration formation caused by *Acanthamoeba* keratitis

be removed from the capillary by an inverted centrifugation step and products visualised by agarose gel electrophoresis.

Results

Of the 285 samples received from 189 patients for whom examination for *Acanthamoeba* was requested, 19 samples from 14 patients yielded one or more positive results. The positive cases are summarised below.

Discussion

Although culture is the traditional and irrefutable method for diagnosis of *Acanthamoeba* keratitis, it requires familiarity with the morphology of cysts and trophozoites of *Acanthamoeba*. Culture may take three–five days before organisms are visualised; plates are incubated for seven days before negative results are reported. Positive cultures may be further delayed if antibiotics with amoebicidal activity have been used in treatment[4].

Comparison of results from culture with real-time PCR assays showed both tests gave positive results, but real-time PCR was more sensitive as well as more rapid to provide a result. This assay has been shown to be both specific and sensitive to detect clinically relevant *Acanthamoeba* strains[3]. Culture of corneal samples is cumbersome because of the need to transport prepared media to the clinic for inoculation. The PCR technique described requires a laboratory equipped for molecular testing and was easily be adapted to real-time amplification. Testing contact lenses and lens cases provides an indirect but satisfactory confirmation of the suspected pathogen and complements direct examination of corneal scrapings or swabs by culture or PCR.

The importance of sample selection was highlighted in this study, in which those five patients from whom multiple samples were submitted all had both positive and negative results from culture and PCR. From the nine patients from whom only a single sample was submitted, three had positive results by both methods, five had positive PCR and negative culture and only one had a positive culture with negative PCR. These results are qualitatively similar to those reported by Pasrischa[2], in which 10 instances of *Acanthamoeba* keratitis were described in 53 consecutive cases of microbial keratitis associated with non-contact lens wearers. No single mode of testing for *Acanthamoeba* was capable of detecting all cases, although most patients had positive results from more than one test method. Clearly, PCR improves diagnostic sensitivity over culture and provides more rapid results. However, the optimal results are obtained from using both PCR and culture from corneal samples, and samples of fluid in lens cases. Our data also confirmed that *Acanthamoeba* infection is associated with contact lens keratitis in nearly 10% of patients tested.

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References

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Positive	Specimen	Culture	Real-time PCR
Case 1	Corneal scraping R & L contact lens solution	Positive after 7 days Positive	Negative Positive
Case 2	Corneal swab Contact lens solution	Negative Positive	Positive Positive
Case 3	Corneal swab	Negative	Positive
Case 4	Corneal scraping Contact lens solution	Positive Positive	Positive Positive
Case 5	Corneal swab	Positive	Negative
Case 6	Corneal scraping	Positive	Positive
Case 7	Corneal swab	Negative	Positive
Case 8	Corneal swab	Negative	Positive
Case 9	Anterior chamber tap	Negative	Positive
Case 10	R & L contact lens solution	Negative	Positive
Case 11	Corneal scraping	Positive	Positive
Case 12	Corneal scraping	Positive	Positive
Case 13	Corneal scraping R & L contact lens solutions	Positive Negative	Positive Negative
Case 14	L contact lens solution R contact lens solution	Positive Negative	Positive Negative