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Effect of storage time and temperature on the detection of *Pseudomonas aeruginosa*, *Acanthamoeba* and Herpes Simplex Virus from corneal impression membranes

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Abstract

The effect of storage time and temperature on the recovery of pathogen DNA from polytetrafluorethylene (PTFE) was investigated. PTFE impression membranes were inoculated with *Pseudomonas aeruginosa*, Herpes Simplex Virus-1 (HSV-1) or *Acanthamoeba* and stored at −70 °C, −20 °C, +4 °C or +35 °C. PCR was performed on days 0, 1, 2, 3, 7 and months 1, 3 and 10 post-inoculation. We found no reduction in the DNA recovery of any of the studied microorganisms for the first 3 days of storage up to +35 °C. For HSV-1 and *P. aeruginosa*, storage for 3 months at +35 °C was associated with a significant reduction in DNA recovery (P<0.001), but not at +4 °C, −20 °C or −70 °C for 1 month for *P. aeruginosa* and for 10 months for HSV-1. *Acanthamoeba* DNA recovery was not affected by any storage parameters (P=0.203). These results will inform the investigation of microbial keratitis where access to microbiological testing is not readily available.

EFFECT OF STORAGE TIME AND TEMPERATURE ON THE DETECTION OF *PSEUDOMONAS AERUGINOSA*, *ACANTHAMOEBA* AND HERPES SIMPLEX VIRUS FROM CORNEAL IMPRESSION MEMBRANES

Corneal ulceration due to microbial keratitis (MK) leads to scarring and neovascularization and is a significant cause of blindness worldwide [1]. The incidence of MK remains higher in low-resource settings relative to more developed countries, and the World Health Organisation (WHO) now recognizes corneal blindness caused by MK as an emerging important cause of visual disability [2]. In most parts of the world, bacteria are the leading pathogens but infections may be caused by fungi, viruses or parasites.

Identifying the causative organism in MK is essential in targeting treatment and ensuring resolution of infection. Corneal samples are traditionally collected from the affected area of cornea with a disposable needle, spatula or blade and requires expertise and proficiency using a slit-lamp biomicroscope. Culture remains the 'gold standard' for isolating and identifying the microorganism; however, recent attempts to improve the diagnostic yield from the investigation of MK have focused on PCR to amplify microbial DNA [3, 4].

In 2015, we developed a method which uses a corneal impression membrane (CIM), made from polytetrafluorethylene (PTFE), that is simply placed on the corneal ulcer before being transported to the laboratory either in brain–heart infusion broth for culture or in a tube for subsequent DNA analysis using PCR [5]. PTFE has been shown previously to provide the greatest cellular yield when compared to polycarbonate, polyethersulfone and polyvinylidene [6]. We have shown that this method has a significantly higher overall isolation rate compared to the conventional scraping method (40.8 versus 26.9 %, respectively, P=0.02), and this is thought to be because the CIM is able to sample a greater surface area of an ulcer [5]. This method, called impression cytology (IC), has been shown to reliably remove epithelial surface cells and increase the detection of viral [7, 8], fungal [9] and *Acanthamoeba* [10] microorganisms in cases of MK. This technique is simple to perform, is less traumatic than the conventional scraping method and does not rely on the need for specialized slit-lamp biomicroscopy by an ophthalmologist, thus lending itself to nurse-led sampling and sampling in low-resource settings.

PCR is becoming more commonplace for detection of microorganisms in cases of microbial keratitis, particularly because it can detect organisms from very small quantities of DNA and can provide more rapid results then culture [11]. This makes it ideally suited for identifying the

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Abbreviations: CIM, corneal impression membrane; Cp, crossing point; MK, microbial keratitis.

causative microorganisms of MK, as retrievable samples from corneal tissue are usually very small.

The majority of studies that focus on PCR to amplify microbial DNA in patients with MK come from developed settings where samples are immediately transferred to the laboratory within in a short period of time. The effects of specimen storage time and temperature on pathogen DNA recovery in MK are currently unknown. This has implications in low-resource settings where samples may need to be transported across large distances to a central laboratory prior to processing, and in the tropics where ambient temperatures are likely to be higher. Identification of parameters that may affect the stability and recovery of pathogen DNA is therefore important. We therefore investigated the effects of storage time and temperature on the recovery of pathogen DNA following inoculation on to CIMs.

The CIM discs were prepared from a sheet of hydrophilic PTFE (Biopore Membrane, Merck Milipore, UK) using a 4 mm punch and autoclaved at 136 °C for 45 min. Stock concentrations of *P. aeruginosa* (10^6 c.f.u. ml^{-1}, clinical isolate) and *Acanthamoeba* cysts (number of cysts 10 μl^{-1} clinical isolate) were made by dilution in molecular grade water. Herpes Simplex Virus type 1 (HSV-1) stock (1000 copies ml^{-1}, clinical isolate) was made by diluting in virus transport medium. The CIM discs were inoculated by pipetting 5 µl of stocks of either *P. aeruginosa, Acanthamoeba* or HSV-1 directly onto the CIM discs. The CIM discs were placed in Eppendorf tubes, air-dried and stored at either −70 °C, −20 °C, +4 °C or +35° in the dark. CIM discs stored at +35 °C were extracted at 0 days, 1 day, 2 days, 3 days, 7 days, 1 month, 3 months and 10 months post-inoculation. CIM discs stored at −70 °C, −20 and 4 °C were extracted at 3 days, 7 days, 1 month, 3 months and 10 months post-inoculation. All experiments were performed in triplicate.

On extraction, 400 µl of HEPES-based buffering agent (Hologic Apitima, Hologic, MA, USA) was added to the CIMs in their primary tubes and vortexed for 10 s. After brief centrifugation, the 400 µl of HEPES-based buffering agent was transferred to a secondary tube. DNA was selectively extracted from the decanted HEPES using the Roche MagNAPure Compact automated extraction platform and the MagNAPure Compact DNA isolation kit I, with a final elution volume of 50 µl (Roche Magna Pure Compact, Roche). A multiplex PCR master mix comprising LC480 Probes Master, primers and fluorescently labelled probes (Eurogentec Ltd) for the detection of HSV-1 (Bennett *et al.* [12]), *Acanthamoeba* (Qvarnstrom *et al.* [13] and Riviere *et al.* [14]) and the bacterial 16S rRNA gene (Patel *et al.* [15]) was prepared. The 16S rRNA gene PCR probe had 100 % homology with *P. aeruginosa.* Twenty microlitre aliquots were pipetted into a 96-well PCR reaction plate and 5 µl of the DNA extract added. The plate was transferred to a real-time PCR instrument (Roche LC480 I) and PCR performed using cycling parameters of 95 °C for 5 min, 45 cycles of 95 °C for 10 s, 60 °C for 45 s, then 72 °C for 1 s, with a final cooling step of 40 °C for 30 s. The crossing point (Cp) value, which is the PCR cycle at which the emitted fluorescence reaches a defined threshold, was recorded for each reaction. When less DNA is recovered, more cycles are needed to reach the crossing point of detection, resulting in a higher Cp value. Statistical analysis was performed using SPSS (version 22). Two-way ANOVA was used to assess any statistically significant effect of storage temperature and time on the recovery of HSV-1, *Acanthamoeba* and *P. aeruginosa* DNA as reflected by the Cp value. Post hoc analysis was carried out using Tukey's post hoc test.

We demonstrated a statistically significant reduction in HSV-1 DNA recovery only when HSV-1-inoculated CIMs were stored for 3 months or longer at +35 °C (P<0.001), (Fig. 1). One out of three of the HSV-1-inoculated CIMs stored at +35 °C for 9 months did not reach the fluorescence threshold. No statistically significant difference in mean Cp values was found for CIMs stored at +4 °C, −20 °C and −70 °C for all time-points, indicating no significant reduction in DNA recovery when HSV-1 inoculated CIMs were stored at these temperatures for at least up to 10 months (Fig. 1). In low-resource settings, where dry ice and ultra-low freezers may not be available, our results indicate that samples may be stored for up to 10 months at 4 °C without any significant reduction in the recovery of DNA. There has been no previous work of this type using corneal samples; however, Jerome *et al.* [16] used a real-time PCR assay to quantitate HSV-1 DNA from swabs placed in PCR medium that were taken from cervical, vulvar, peri-anal and oral sites for women and penile, peri-anal and oral sites for men. Their results demonstrated that HSV-1 DNA remains quantitatively stable over 16 months when stored as an unextracted specimen at −20 °C or when stored at +4 °C as extracted DNA. Comparison of the HSV-1 DNA levels before or after storage showed no reduction over 16 months. Our results appear to reflect this up to 10 months, but in addition demonstrate that HSV-1 DNA levels remained stable on the CIM disc without the need for a buffered transport medium. Furthermore, our results suggest a 3-month window period in which CIM samples obtained from clinically suspected HSV keratitis patients may be safely stored at ambient temperature without any significant degradation of DNA.

We found no statistically significant differences in mean Cp values between storage temperatures (P=0.140) and storage time (P=0.561) for *Acanthamoeba.* This is in keeping with what is already known about the highly resistant structure of *Acanthamoeba. Acanthamoeba* are more commonly seen as cysts in keratitis. In addition, it is thought that *Acanthamoeba* encyst when they undergo shock. This is what is thought to occur when they are removed from the corneal epithelium during corneal sampling in MK [17]. The cell wall of a mature cyst has two layers: a rounded or wrinkled outer layer (the ectocyst), composed of protein, and a rounded or stellate inner layer (the endocyst) consisting mostly of cellulose. Therefore, cysts are very resistant to physical and chemical agents such as chlorine and most

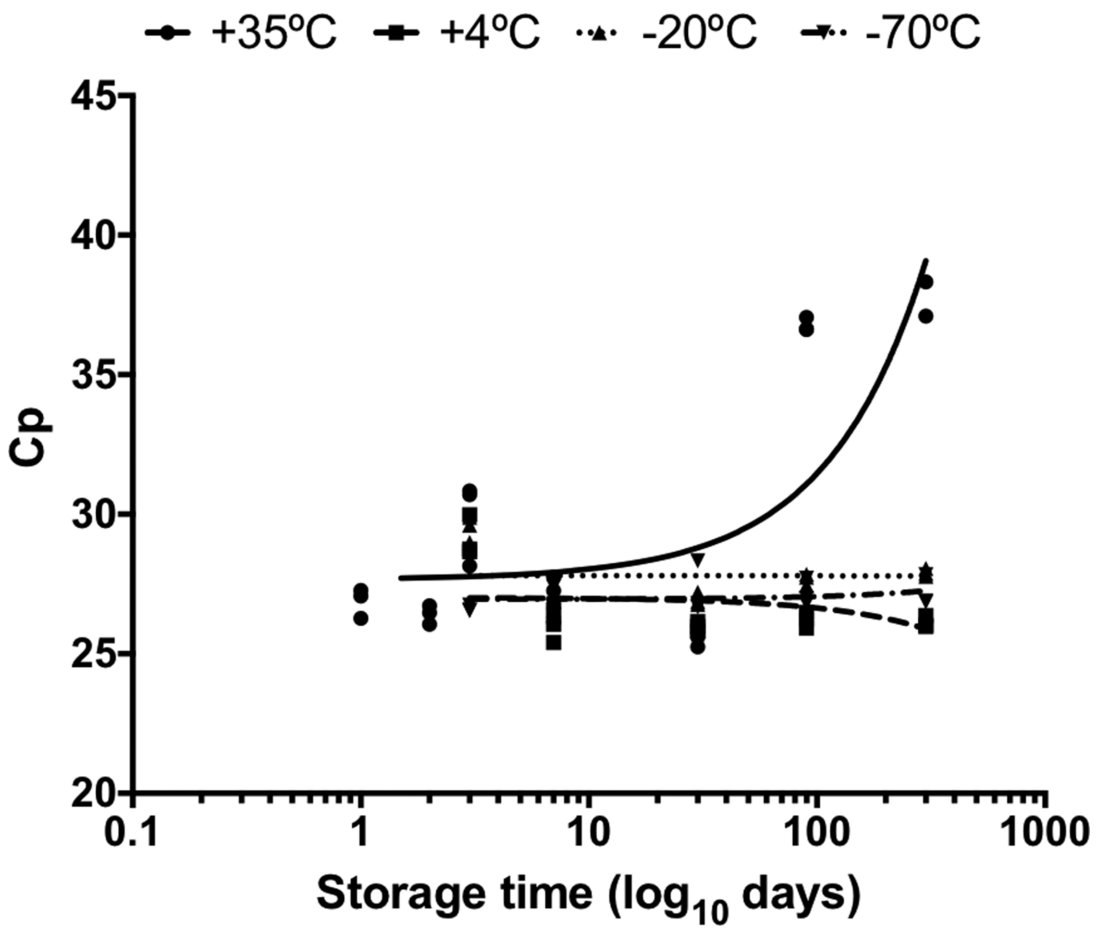

Fig. 1. The effect of storage temperature and time on HSV-1 DNA recovery from inoculated PTFE CIMs. Points represent individual experiments. Experiments were performed in triplicate. 35 °C (R^2 : 0.68, *P*=0.01); 4 °C (R^2 : 0.12, *P*=0.57); −20 °C (R^2 : 4.19e-005, *P*=0.99); −70 °C (R^2 : 0.23, *P*=0.90). Cp (crossing point).

biocides and antibiotics, as well as temperature [18]. This highly resistant structure and difficulties associated with cyst lysis may explain the number of samples that did not reach the defined fluorescence threshold (one out of three of the *Acanthamoeba*-inoculated CIMs stored at +35 °C, +4 °C and −70 °C for 7 days, respectively; one out of three of the *Acanthamoeba*-inoculated CIMs stored at +35 °C and +4 °C for 1 month, respectively; two out of three of the *Acanthamoeba*-inoculated CIMs stored at +35 °C for 3 months; and one out of three of the *Acanthamoeba*-inoculated CIMs stored at +35 °C for 9 months). The number of inoculated *Acanthamoeba* cysts in this study reflects the number that would be seen on a CIM in clinical practice, and is a lower sample load then for viruses or bacteria. The increase in sampling variance seen with *Acanthamoeba* (mean Cp variance between triplicate experiments was 4.6, 0.4 and 0.6 for *Acanthamoeba*, HSV and *P. aeruginosa*, respectively) is thought to be secondary to this lower sample load [19]. To our knowledge, no previous studies have looked at DNA recovery of *Acanthamoeba.*

We demonstrated a significant reduction in the recovery of bacterial DNA only when *P. aeruginosa*-inoculated CIMs were stored at +35 °C for 1 month or longer (*P*<0.002), Fig. 2. In addition, we demonstrated a significant reduction in the recovery of *P. aeruginosa* DNA only following storage for 3 months at +4 °C, −20 °C and −70 °C compared to those CIMs extracted on day 0 (*P*<0.029), and this was to a much lesser degree than that seen at storage temperatures of 35 °C, indicating up to a 1-month time frame in which samples should be processed. Post hoc analysis for storage temperature demonstrated no statistically significant difference (*P*=0.98) between mean Cp values for −20 °C and −70 °C, which has significant cost implications for sample storage. One out of three of the *P. aeruginosa*-inoculated CIMs stored at +4 °C and −20 °C for 3 months, respectively, and one out of three of the *P. aeruginosa*-inoculated CIMs stored at +35 °C and −70 °C for 9 months, respectively, did not reach the defined fluorescence threshold. To date, no other studies have looked specifically at the effect of temperature and storage time on *P. aeruginosa* DNA recovery.

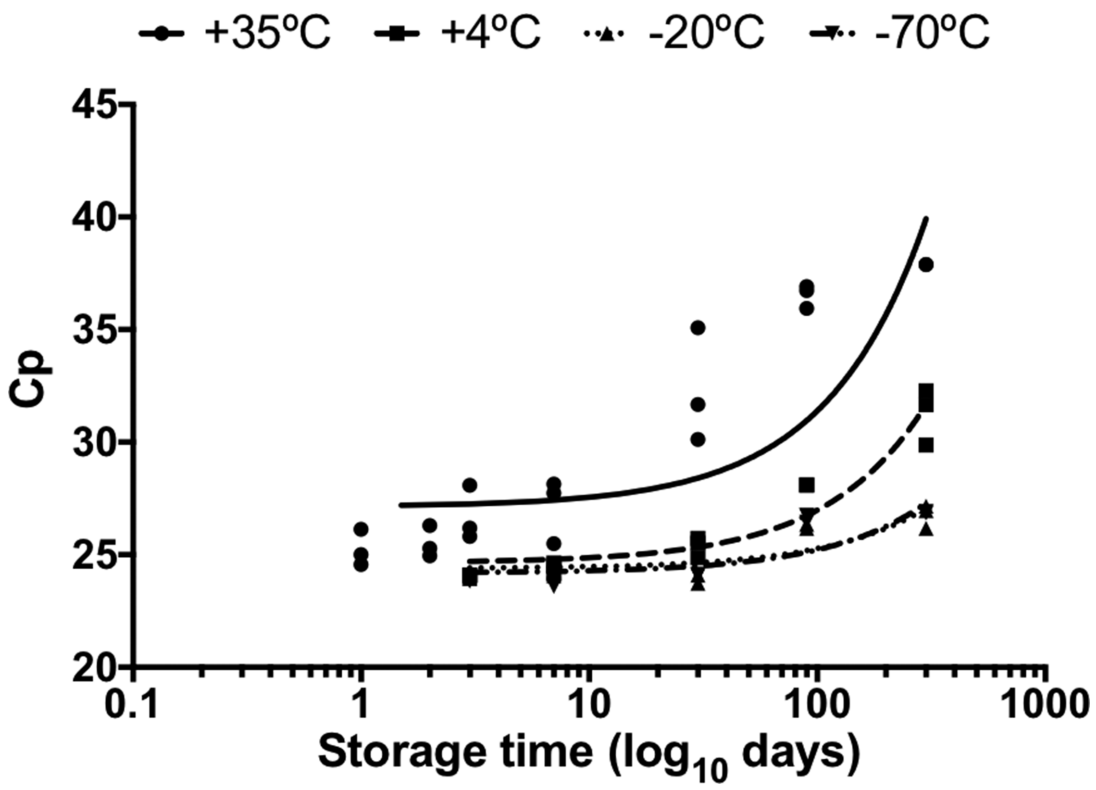

Fig. 2. The effect of storage temperature and time on *P. aeruginosa* DNA recovery from inoculated PTFE CIMs. Experiments were performed in triplicate. 35 °C (R^2 : 0.68, *P*=0.01); 4 °C (R^2 : 0.93, *P*=0.007); −20 °C (R^2 : 0.77, *P*=0.05); −70 °C (R^2 : 0.70, *P*=0.08); Cp (crossing point).

In this study, we demonstrate that both storage time and temperature have statistically significant effects on the recovery of both HSV-1 and *P. aeruginosa* DNA, but not *Acanthamoeba* DNA. Importantly for diagnostic purposes in tropical settings, our results suggest there is no reduction in DNA recovery for any of the studied microorganisms for the first 3 days of storage up to +35 °C. When stored at +4 °C, −20 °C or −70 °C, there was no loss of 16S bacterial DNA up to 1 month and HSV-1 DNA up to 10 months. Further work may be required to assess whether recovery of DNA is dependent on the inoculum dosage. Although additional work is required to extrapolate this work to other recognized ocular pathogens such as *Staphylococcus aureus*, we would expect results comparable to *P. aeruginosa*. Further work is, however, required to confirm these findings in positive CIM samples in clinical practice.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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