

Induced encystment improves resistance to preservation and storage of *Acanthamoeba castellanii*

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SUMMARY

Several conditions that allow the preservation, storage and rapid, efficient recovery of viable *Acanthamoeba castellanii* organisms were investigated. The viability of trophozoites (as determined by time to confluence) significantly declined over a period of 12 months when stored at -70°C using dimethyl sulfoxide (DMSO; 5 or 10%) as cryopreservant. As *A. castellanii* are naturally capable of encystment, studies were undertaken to determine whether induced encystment might improve the viability of organisms under a number of storage conditions. *A. castellanii* cysts stored in the presence of Mg^{2+} at 4°C remained viable over the study period, although time to confluence was increased from approximately 8 days to approximately 24 days over the 12-month period. Storage of cysts at -70°C with DMSO (5 or 10%) or 40% glycerol, but not 80% glycerol as cryopreservants increased their viability over the 12-month study period compared with those stored at room temperature. Continued presence of Mg^{2+} in medium during storage had no adverse effects and generally improved recovery of viable organisms. The present study demonstrates that *A. castellanii* can be stored as a non-multiplicative form inexpensively, without a need for cryopreservation, for at least 12 months, but viability is increased by storage at -70°C .

Key words: *Acanthamoeba*, preservation, storage, cyst, trophozoite.

INTRODUCTION

Acanthamoeba species are normally free-living protozoa, their transmission and spread does not depend on a host and they are found almost ubiquitously in the environment (reviewed by Marciano-Cabral and Cabral, 2003). Several species are pathogenic, causing granulomatous amoebic encephalitis (GAE) and cutaneous acanthamoebiasis in immunocompromised or immunosuppressed hosts, and amoebic keratitis (AK) in immunocompetent individuals (reviewed by Marciano-Cabral and Cabral, 2003; Schuster and Visvesvara, 2004). As the importance of this pathogen is recognized there has been an increase in the number of studies, particularly those investigating new antimicrobials, that may improve treatment (Schuster and Visvesvara, 1998; Mattana *et al.* 2004; McBride *et al.* 2007; Henriquez *et al.* 2008).

The ability to preserve protozoa in a non-actively dividing state has a number of advantages. Firstly, it reduces the material and labour costs of continued culture and passage, secondly it reduces the chance of selection for culture-adapted organisms observed during continued tissue culture and it also allows the

possibility to maintain more than one isolate in a single location. Furthermore, it may facilitate transport of isolates without regard to time. The cryopreservation of *Acanthamoeba* and other free-living amoebae has been previously described (Seo *et al.* 1992; John *et al.* 1994; John and John, 1996; Alejandro-Aguilar *et al.* 1998; Gonzalez-Robles *et al.* 2001). The slow cooling (overall $0.7^{\circ}\text{C}/\text{min}$) of free-living amoeba trophozoites, *Acanthamoeba culbertsoni*, *A. polyphaga*, *Naegleria fowleri* and *N. gruberi*, with 7.5% dimethylsulfoxide (DMSO) or 7.5% glycerol produced 2–39% viable trophozoites after 60 days in liquid nitrogen (Seo *et al.* 1992). John *et al.* (1994) reported that recovery of *A. castellanii* trophozoites decreases with longer storage times at -70°C with 12% DMSO, 20% heat-inactivated bovine calf serum and 4% glucose with the greatest reduction during the first year of storage (John and John, 1996). Alejandro-Aguilar *et al.* (1998) found that the viability of *A. castellanii* trophozoites increased when stored with 10% DMSO in liquid nitrogen if an equilibrium temperature of 4°C was used.

As we found that our ability to recover viable trophozoites from cryopreservation reduced with time we sought an alternative method. *Acanthamoeba* exist as active trophozoites or dormant cysts. Encystment occurs when the organism is exposed to adverse environmental conditions such as absence of nutrients, biocides, fluctuations in temperature and

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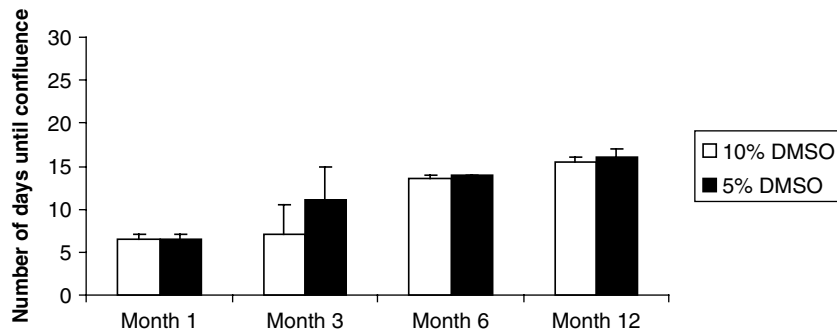


Fig. 1. The average time (days) until confluence for *Acanthamoeba castellanii* trophozoites stored in RM with 10% or 5% DMSO at -70°C . The length of time to reach confluence increased the longer time spent in storage for all samples from 6.5 to 16 days. The data represent the mean of 2 replicate experiments.

pH (reviewed by Schuster, 2002). Encystment can be induced in the laboratory by the addition of MgCl_2 to the medium (reviewed by Schuster, 2002). We hypothesized that encystment of *Acanthamoeba* may increase their viability during cryopreservation or eliminate the need for it completely.

Herein, a number of different storage conditions for *A. castellanii* trophozoites and cysts are compared. The effectiveness of each condition was determined via microscopical observations of the time required to reach confluence.

MATERIALS AND METHODS

Maintenance of *A. castellanii* trophozoites

Acanthamoeba castellanii (Neff strain) was kindly donated by Keith Vickerman (Glasgow, UK). Trophozoites were routinely grown in RM containing 2% mycological peptone (Sigma, Poole, UK) and 0.9% maltose (Sigma, Poole, UK), supplemented with 125 μg penicillin/streptomycin and 2.5 $\mu\text{g}/\text{ml}$ amphotericin B (Sigma, Poole, UK) as previously described (McBride *et al.* 2005). They were incubated until confluent at room temperature in 75- cm^2 tissue-culture flasks, followed by either subculture or harvest by mechanical detachment.

Preparation of cysts

The RM was removed from confluent 75- cm^2 tissue-culture flasks and replaced by 10 ml of encystment medium (EM) (20 mM Tris-HCl, pH 8.8, 100 mM KCl, 8 mM MgSO_4 , 0.4 mM CaCl_2 , 1 mM NaHCO_3 – all reagents from Sigma) (Neff *et al.* 1964) and monitored until cysts had formed (2–3 days). The cysts were then collected by centrifugation at 1200 g for 5 min resuspended in (EM) and counted using a haemocytometer. Cysts were adjusted to $3 \times 10^5/\text{ml}$, added to microcentrifuge tubes (ThermoElectron, UK) and centrifuged at 20 000 g for 5 min. The EM was then removed and the cysts were resuspended in 1 ml of the appropriate medium for storage: (1) EM or RM with (a) 10% DMSO, (b) 5% DMSO, (c) 2.5%

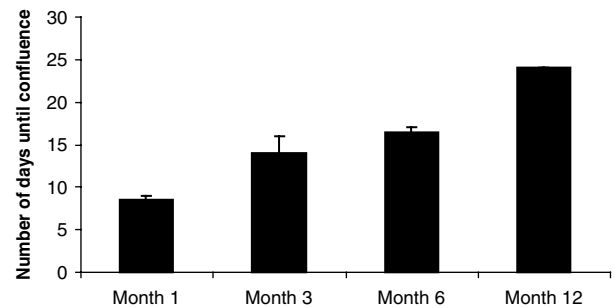


Fig. 2. The average time (days) until confluence for *Acanthamoeba castellanii* cysts stored in EM at 4°C . The length of time to reach confluence increased the longer time spent in storage from 8.5 to 24 days. The data represent the mean of 2 replicate experiments.

DMSO, (d) 80% glycerol, (e) 40% glycerol and (f) 20% glycerol or (2) EM alone. EM and RM suspensions with glycerol or DMSO were then transferred to a cryopreservation tube and stored at -70°C and EM alone was placed at 4°C for 1, 3, 6 and 12 months.

Preparation of trophozoites

Trophozoites were collected from newly confluent cultures by mechanical disruption followed by centrifugation at 1200 g for 5 min and resuspended in RM. Then 3×10^5 trophozoites in 1 ml of suspension were added to microcentrifuge tubes and centrifuged at 1200 g for 5 min. The RM was then removed and the trophozoites were resuspended in RM, counted using a haemocytometer and adjusted to $3 \times 10^5/\text{ml}$ in the appropriate medium for storage: RM with (a) 10% DMSO, (b) 5% DMSO, (c) 2.5% DMSO, (d) 80% glycerol, (e) 40% glycerol and (f) 20% glycerol. The trophozoite suspensions were then transferred to a cryopreservation tube (Nunc, Denmark) and stored at -70°C for 1, 3, 6 and 12 months.

Recovery of *Acanthamoeba*

After the appropriate length of time in storage (1, 3, 6 and 12 months) each cryovial was thawed slowly at

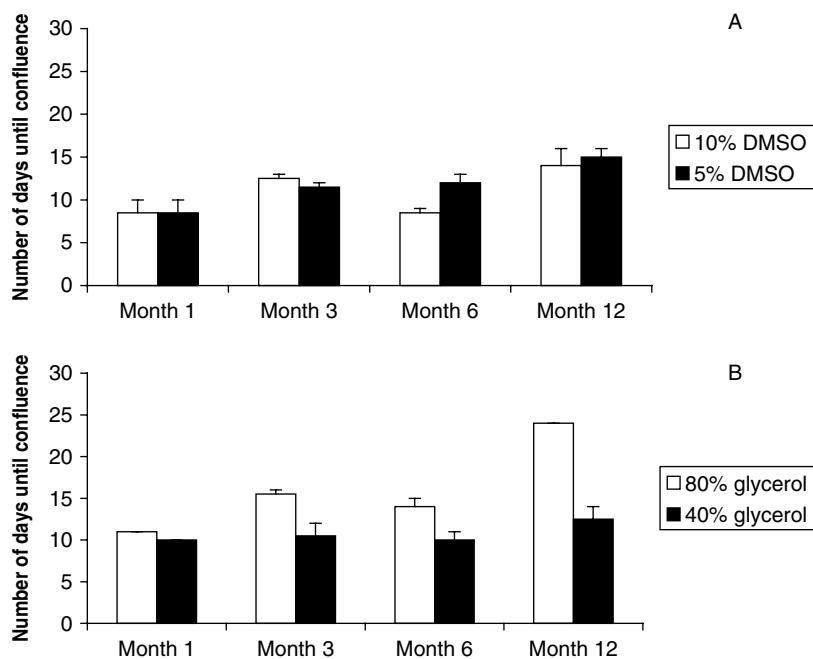


Fig. 3. The average time (days) until confluence for *Acanthamoeba castellanii* cysts stored at -70°C in RM with (A) 10% or 5% DMSO and (B) 80% or 40% glycerol. (A) The length of time to reach confluence increased for cysts stored with RM and 5% DMSO the longer time spent in storage from 8.5 to 15 days whereas cysts stored with 10% DMSO varied from 8.5 to 14 days over the 12-month period. (B) The length of time until confluence remained constant for cysts stored in 40% glycerol and RM (10–12.5 days) whereas cysts stored with 80% glycerol increased after from 11 to 24 days. The data represent the mean of 2 replicate experiments.

room temperature and then transferred to a micro-centrifuge tube. Cells were collected by a 5-min centrifugation at 13 000 rpm and 4°C , resuspended in 5 ml of RM the transferred to 25 cm^2 flasks. All samples were monitored every 24 h until the flasks containing either trophozoites or cysts, which revert back to trophozoites in RM, reached 100% confluency.

RESULTS

A. castellanii trophozoites and cysts were stored under various conditions to determine their optimal long-term storage method. At 1, 3, 6 and 12 months after being placed in storage, *A. castellanii* were placed in RM until 100% confluency was reached (Figs 1–4). This demonstrated their viability. Both *Acanthamoeba* trophozoites and cysts remained viable in all storage media, with the exception of those stored in media containing 20% and 10% glycerol (results not shown). The length of time to reach 100% confluency increased for *A. castellanii* trophozoites stored in RM with 10% or 5% DMSO at -70°C from 6.5 to 16 days as the length of time to storage increased (Fig. 1). The time to 100% confluency also increased for *A. castellanii* cysts stored in EM at 4°C from 8.5 to 24 days as the time in storage increased from 1 to 12 months (Fig. 2).

The length of time to 100% confluency increased for *A. castellanii* cysts stored in RM and 5% DMSO with length of time spent in storage (from 8.5 to 15

days). However, at a DMSO concentration of 10% the length of time to confluence varied throughout the entire study, with an increase from 1 month to 3 months (8.5 to 12.5 days), then decreasing after 6 months (8.5 days) and finally increasing after 12 months (14 days) (Fig. 3). For cysts stored in 40% glycerol and RM the length of time to confluence remained constant (10–12.5 days), whereas for cysts stored in 80% glycerol the time required to reach trophozoite confluence increased (from 11 to 24) over the 12-month study period (Fig. 3).

The average time until confluence for *A. castellanii* cysts stored at -70°C in EM and in 5% or 10% DMSO varied from 9 to 13 days over the 12-month period (Fig. 4). However, for *A. castellanii* cysts stored in EM with 80% glycerol the time to confluence increased over the study period from 13 to 24 days and cysts stored in 40% glycerol remained constant at 10–12.5 days (Fig. 4).

DISCUSSION

Cryopreservation of *Acanthamoeba* species has been previously described in a number of studies (Seo *et al.* 1992; John *et al.* 1994; John and John, 1996; Alejandre-Aguilar *et al.* 1998; Gonzalez-Robles *et al.* 2001). A solution containing 10% DMSO had previously been shown to produce the highest number of viable *A. castellanii* trophozoites after storage in liquid nitrogen for up to 210 days (Alejandre-Aguilar *et al.* 1998). Other studies have also demonstrated

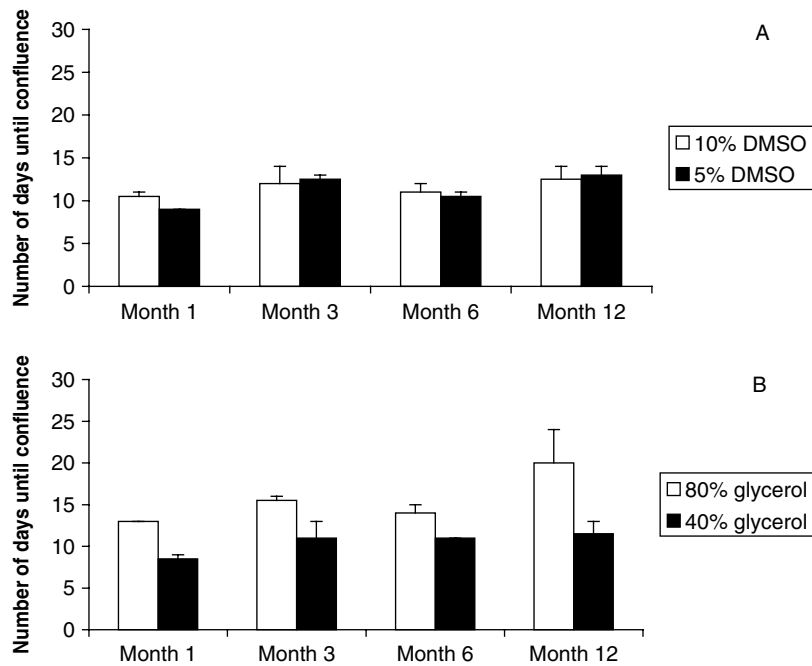


Fig. 4. The average time (days) until confluence for *Acanthamoeba castellanii* cysts stored at -70°C in EM with (A) 10% or 5% DMSO and (B) 80% or 40% glycerol. (A) The length of time for cysts in 5% or 10% DMSO varied from 9 to 13 days over the 12-month period. (B) Cysts stored in EM with 80% glycerol took a lot longer to reach confluence (13–24 days) than cysts stored in 40% glycerol (10–12.5 days). The data represent the mean of 2 replicate experiments.

that the viability of *A. castellanii* trophozoites decreases during the first year of storage in the presence of 12% DMSO at -70°C (John *et al.* 1994). The studies undertaken herein, to investigate alternative means of storage, were instigated by similar anecdotal observations in our laboratory.

Acanthamoeba transform into cystic stages under adverse conditions and are resistant to biocides, chlorination and antibiotics. Cysts have been shown to give rise to viable trophozoites after 24 years in storage at 4°C (Mazur *et al.* 1995; Marciano-Cabral and Cabral, 2003), but their viability has not previously been assessed relative to cryopreserved trophozoites. The results of the present study confirm that viability as determined by time to confluence is reduced in stabilates stored with DMSO or glycerol as cryopreservant following only 1 month of storage at -70°C . However, cysts in EM stored at 4°C remain viable for in excess of 1 year although time to reach confluence is increased with time in storage, up to 12 months. Specifically, time to reach confluence increased from 8.5 to 24 days over this time-period. Viability could be markedly improved by storage at -70°C in the presence of either DMSO or glycerol as cryopreservant. Notably, those stored in 40% glycerol remained relatively constant throughout the whole time-period, reaching confluence at 8.5 days after the first month of storage and between 10 and 12.5 days for time-periods up to and including 1 year. Similar results were obtained using 5% and 10% DMSO as cryopreservant. The presence of Mg^{2+} used to induce encystment was not necessary in the

cryo-medium, but did not have an adverse affect on amoeba viability.

Overall these studies demonstrate that *A. castellanii* are best stored as cyst stages rather than as trophozoites. In this state they can be easily and inexpensively preserved for at least 12 months without the need for cryopreservation. However, preservation can be improved when stored -70°C with cryo-preservant and this refinement is recommended for long-term storage where facilities exist.

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