RESEARCH NOTE

VIABILITY OF PATHOGENIC *NAEGLERIA* AND *ACANTHAMOEBA* ISOLATES DURING 10 YEARS OF CRYOPRESERVATION

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Abstract. This is a follow-up report on the viability of pathogenic *Naegleria fowleri*, *Naegleria australiensis* and *Acanthamoeba castellanii* isolates during 5 to 10 years of cryopreservation at –70°C. The greatest decrease in viability occurred with *N. fowleri* and the least occurred with *N. australiensis*. At 10 years of cryostorage, viability was 21% for *N. fowleri*, 32% for *A. castellanii* and 51% for *N. australiensis.*

The cryogenic preservation of live cells eliminates the need for continuous passage which invariably leads to genetic variation. With *Naegleria fowleri* Carter, 1970, a free-living amoeba which causes primary amoebic meningoencephalitis in humans (reviewed by John 2005), there is a loss of virulence associated with prolonged axenic cultivation (Wong et al. 1977). Previously, we published a procedure for the optimal cryopreservation of pathogenic *Naegleria* and *Acanthamoeba* isolates (John et al. 1994) and a 5-year follow-up report (John and John 1996). The present report describes the viability of *N. fowleri*, *Naegleria australiensis* De Jonckheere, 1981 and *Acanthamoeba castellanii* Douglas, 1930 during 5 to 10 years of cryopreservation.

The amoebae used in this study were *A. castellanii* (EI-212), isolated from the environment in Oklahoma (John and Howard 1995); *N. australiensis* (PP-397), isolated from the environment in Australia (De Jonckheere 1981) and kindly supplied by Johan De Jonckheere; and *N. fowleri* (LEE), isolated from patient cerebrospinal fluid in Virginia (Duma et al. 1969) and originally supplied by Clifford Nelson. The EI-212 strain of *A. castellanii* was identified by morphology and by indirect immunofluorescence with antisera produced in immunized rabbits. The PP-397 strain of *N. australiensis* is maintained by the American Type Culture Collection (Manassas, Virginia) under the strain designation ATCC 30958. All strains were pathogenic to mice by intranasal instillation and produced fatal infections involving the central nervous system.

Amoebae were grown axenically in Mix amoeba medium (John 1993), an equal mixture of Balamuth's (1964) and Nelson's (Nelson and Jones 1970; for composition see Weik and John 1977) media supplemented with 4% bovine calf serum and 1 µg/ml hemin. Amoebae were cultivated in 25-cm^2 polystyrene tissue-culture flasks (Corning Glass Works, Corning, New York). Cultures were inoculated with 1×10^5 amoebae and incubated at 37°C.

Exponential growth-phase amoebae were used for freezing and were rinsed twice with fresh Mix medium while in the tissue-culture flasks and then suspended at 1×10^6 amoebae/ml in the freezing medium which consisted of 12% dimethylsulfoxide (DMSO), 20% heat-inactivated bovine calf serum, 4% glucose, in Mix amoeba medium (John et al. 1994). One-ml quantities of amoebae in freezing medium were dispensed in cryogenic vials (Corning), allowing to equilibrate at 23°C (room temperature) for 30 min, placed at –20°C for 1h and stored at –70°C in an ultra-low temperature freezer (Harris Manufacturing Co., North Billerica, Massachusetts).

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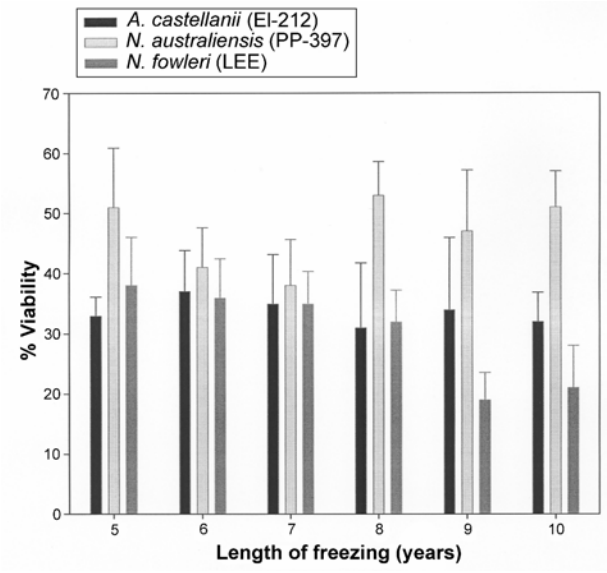

Fig. 1. Viability of pathogenic free-living amoebae during years 5–10 of cryopreservation.

Thawing of the amoebae was accomplished by swirling the cryovials in a 37°C waterbath. Viability was determined by exclusion of 0.4% Congo red prepared in deionized water. The Congo red solution was mixed with equal volumes of amoebae in freezing medium and viability was assessed by light microscopy. Viable amoebae appeared pale blue and nonviable cells were reddish brown. Each percent viability illustrated in Fig. 1 is the average viability of 1,200 amoebae (three counts of 200 cells for each of two cryovials). Viability was confirmed by growth of the amoebae in Mix medium.

The conditions of cryopreservation used in this study were ones that we described in an earlier publication (John et al. 1994) and were conditions that produced the average best viabilities for the three pathogenic isolates examined, namely, *A. castellanii*, *N. australiensis*, and *N. fowleri*. The present study is a 5 to 10-year follow-up of the viability of the three species.

Fig. 1 illustrates the percent viabilities of the three species at yearly intervals during the 5 to 10 years of freezing. At the end of 10 years of cryostorage, viability was 21% for *N. fowleri*, 32% for *A. castellanii* and 51% for *N. australiensis*. Viability was confirmed by growth of amoebae in Mix amoeba medium.

In our previous paper (John and John 1996), we reported that the greatest decrease in viability occurred during the first year of freezing and was 10-fold greater than the average yearly decrease during years 2–5. At one year of cryopreservation, viability for the three species was 47%, 39% and 53% for *N. fowleri*, *A. castellanii* and *N. australiensis*, respectively.

The greatest decrease in viability between years 5 and 10 occurred with *N. fowleri* and was 45%. *Acanthamoeba castellanii* exhibited a 3% decrease and *N. australiensis* exhibited no decrease in viability between years 5 and 10. The overall decrease in viability between years 1 and 10 of cryopreservation was 55% for *N. fowleri*, 18% for *A. castellanii* and 4% for *N. australiensis*.

Of the few reports describing cryopreservation of free-living amoebae, most have examined viability after several days or a few weeks of freezing (Kilvington and White 1991, Simione and Daggett 1976). However, there are two reports that describe viability after a somewhat longer period of cryostorage. Alejandre-Aguilar et al. (1998) reported 75% viability for pathogenic *Acanthamoeba culbertsoni* trophozoites stored in liquid nitrogen for 210 days (7 months). Brown and Day (1993) observed 46% viability for nonpathogenic *Naegleria gruberi* trophozoites maintained in liquid nitrogen for 2 years. Both groups used DMSO as a cryoprotectant, 10% in the former and 5% in the latter, as did we, at 12%.

Subsequent to the initial freezing and cryostorage of the large number of amoebae-containing cryogenic vials, the results of which we have reported here and in two prior papers, we have observed that viability is improved if the step involving the equilibration of vials at 23°C (room temperature) for 30 minutes (see above) is eliminated. Consequently, we no longer include this step in the freezing procedure and would advise others using our protocol to do likewise. Additionally, a Cell Freezer (Nalgene Cryo 1°C Freezing Container, Nalgene cat. no. 5100-0001) may be used to eliminate the –20°C step. The Cell Freezer controls the rate of freezing to approximately 1°C/min.

The present study utilized a –70°C ultra-low freezer in which to store the amoebae, thus eliminating the need for liquid nitrogen and cryogenic dewars. Considering we have not used liquid nitrogen and a temperature of –196°C for storage, the results are excellent for 10 years of freezer storage at –70°C for trophozoites of *N. fowleri*, *N. australiensis* and *A. castellanii*.

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