FOLIA PARASITOLOGICA 41: 110–114, 1994

Cryopreservation of pathogenic free-living amebae

D. T. John[1], **P. L. Eddy**[2] and **R. A. John**[3]

[1] Department of Biochemistry and Microbiology, Oklahoma State University, College of Osteopathic Medicine, 1111 West 17th Street, Tulsa, Oklahoma 74107, USA;
[2] University of Oklahoma, Health Sciences Center, Oklahoma City, Oklahoma 73190, USA;
[3] Chapman Institute of Medical Genetics, Children's Medical Center, Tulsa, Oklahoma 74135, USA

Key words: cryopreservation, amebae, *Naegleria*, *Acanthamoeba*

Abstract. A variety of conditions of cryopreservation were evaluated in order to define a single procedure for freezing the amebae of pathogenic *Naegleria* and *Acanthamoeba*. The average best conditions for freezing the three species studied were: $1x10^6$ exponentially growing amebae/ml of freezing medium consisting of 12% dimethylsulfoxide, 20% heat-inactivated bovine calf serum, 4% glucose, in Mix ameba medium; 30 min equilibration at 23° C (room temperature), followed by 60 min at –20° C, with storage at –70° C. Under these conditions viability after 1 month of freezing was 53% for *Acanthamoeba castellanii*, 64% for *Naegleria fowleri*, and 66% for *Naegleria australiensis*. After 12 months of freezing, viability was 39% for *A. castellanii*, 47% for *N. fowleri*, and 53% for *N. australiensis*.

Pathogenic free-living amebae belonging to the genera *Acanthamoeba* and *Naegleria* cause serious infection of the eye and the central nervous system (CNS) (reviewed by John 1993). Species of *Acanthamoeba* may produce a chronic CNS infection known as granulomatous amebic encephalitis or an eye infection referred to as *Acanthamoeba* keratitis. *Naegleria fowleri* is responsible for a rapidly fatal infection of the CNS called primary amebic meningoencephalitis.

Cryopreservation has become a routine feature of the microbiology research laboratory. Unfortunately, there are only a few reports that deal with the cryopreservation of pathogenic free-living amebae (Simione and Daggett 1976, Kilvington and White 1991). The cysts of free-living amebae are more readily frozen than the trophozoites; however, by using a cryoprotectant amebae may be frozen and stored at ultralow temperatures. The purpose of this study was to evaluate a variety of conditions of cryopreservation of amebae and develop a single procedure for freezing pathogenic *Naegleria* and *Acanthamoeba*.

MATERIALS AND METHODS

Amebae and cultivation. The amebae used in this study were *Acanthamoeba castellanii* (EI-212), isolated from the environment in Oklahoma; *Naegleria australiensis* (PP-397), isolated from the environment in Australia and kindly supplied by Johan De Jonckheere; and *Naegleria fowleri* (LEE), isolated from patient cerebrospinal fluid in Virginia and originally supplied by Clifford Nelson. All are pathogenic to mice by intranasal instillation and produce fatal CNS infection.

Amebae were grown axenically in Mix ameba medium (John 1993), an equal mixture of Balamuth's (1964) and Nelson's (1970) media consisting of 0.55% Panmede liver digest, 0.50% proteose peptone, 0.25% yeast extract, and 0.30% glucose in Page's ameba saline (0.12 g NaCl, 0.004 g $MgSO_4 . 7H_2O$, 0.004 g $CaCl_2.2H_2O$, 0.142 g Na_2HPO_4, 0.136 g KH_2PO_4 per liter of distilled water) (Page 1988) supplemented with 4% bovine calf serum and 1 μg/ml hemin. Amebae were cultivated in 25 cm^2 polystyrene tissue-culture flasks (Corning Glass Works, Corning, New York). Cultures were inoculated with $1x10^5$ amebae and incubated at 37° C.

Freezing and viability. Exponential growth-phase amebae were used for freezing and were rinsed twice with fresh Mix ameba medium while in the tissue-culture flasks and then suspended in the freezing medium which was different for each experiment and so is described in the footnotes of each table. One-ml quantities of amebae in freezing medium were dispensed in cryogenic vials (Corning), placed at –20° C for 1h, and stored at –70° C in an ultralow temperature freezer (Harris Manufacturing Co., North Billercia, Massachusetts).

The cryoprotectants that we tested were dimethylsulfoxide (DMSO), glycerol, hydroxyethyl starch (HES), polyvinylpyrrolidone m.w. 10,000 (PVP-10), polyvinylpyrrolidone m.w. 40,000 (PVP-40), and bovine serum, both fetal and calf. All except bovine serum were purchased from Sigma Chemical Co. (St. Louis, Missouri). Fetal bovine serum (FBS) and bovine calf serum (BCS) were purchased from KC Biological, Inc. (Lenexa, Kansas).

Amebae were rapidly thawed by placing the cryovials in a 37° C water bath. Viability was determined by exclusion of 0.4% Congo red prepared in deionized water. The Congo red solution was mixed with equal volumes of amebae in freezing medium and viability was assessed by light microscopy. Viable amebae appe-

ared as pale blue and nonviable cells were reddish brown. We also evaluated viability staining with 0.4% trypan blue and 1% neutral red and found Congo red to be superior for distinguishing live and dead amebae. Each percent viability listed in the tables and figures is the average viability for 1,800 amebae (three counts of 200 cells for each of three cryovials).

RESULTS AND DISCUSSION

The cryogenic preservation of live cells eliminates the need for continuous passage which invariably leads to genetic variation. With the pathogenic free-living amebae there is a loss of virulence associated with prolonged axenic cultivation. In this report we examine a number of conditions of cryopreservation of amebae in order to describe a single procedure for freezing pathogenic *Naegleria* and *Acanthamoeba*. The conditions that we evaluated were the kinds and concentrations of cryoprotectants, kinds and concentrations of bovine serum, cell concentration, glucose, freezing schedules, and phase of ameba growth.

The purpose of a cryoprotectant is to minimize cell damage caused by the formation of ice crystals within the cell. As the water outside the cell freezes the salt concentration increases and osmotic flow draws water out of the cell allowing it to dehydrate and not form ice crystals. Although it is not known how cryopreservatives actually protect cells, they are able to freely penetrate membranes, dissolve electrolytes, and prevent water from freezing at 0° C (Anonymous 1986). Of the cryopreservatives examined only DMSO was able to effectively protect all

Table 1. Viability of pathogenic free-living amebae frozen with various cryoprotectans [a].

	Amebae			
Protectant[b]	*A. castellanii* (EI-212)	*N. australiensis* (PP-397)	*N. fowleri* (LEE)	Average of 3 species
10% DMSO	47.4±8.1[c]	31.3±6.0[c]	45.1±8.8[c]	41.3[c]
10% glycerol	37.9±7.4	1.0±0.3	1.6±0.6	13.5
90% FBS[d]	28.5±11.0	0.1±0.1	3.9±1.0	10.8
6% HES	23.2±0.7	0.2±0.2	1.0±0.6	8.1
6% PVP-10	22.6±6.0	0.7±0.4	0.5±0.3	7.9
6% PVP-40	16.8±1.2	0.2±0.2	0.2±0.2	5.7
90% medium[e]	2.1±1.1	0	0.8±0.4	1.0

[a] Percent viability was determined 1 month after freezing.
[b] The freezing medium consisted of the following: % cryoprotectant (see Materials and Methods), 40% FBS (heat-inactivated), and Mix ameba medium making the balance of 100% with a cell concentration of 1x10[6] amebae/ml.
[c] Greatest % viability.
[d] Freezing medium was 90% fetal bovine serum plus 10% Mix ameba medium.
[e] Freezing medium was 90% Mix ameba medium plus 10% FBS.

Table 2. Viability of pathogenic free-living amebae frozen with various concentrations of DMSO [a].

	Amebae			
% DMSO[b]	*A. castellanii* (EI-212)	*N. australiensis* (PP-397)	*N. fowleri* (LEE)	Average of 3 species
0	14.3±3.3	0.6	2.1±2.3	5.5
2	15.8±10.2	9.9±0.9	6.4±2.4	10.7
4	23.8±8.4	8.2±6.9	15.7±1.8	15.9
8	25.2±4.8	29.3±1.6	33.7±4.0	29.4
10	25.9±4.9	37.2±5.3	31.7±5.9	31.6
12	25.1±24.9	43.3±6.3[c]	38.2±5.3[c]	35.5[c]
16	33.2±15.0[c]	39.3±5.0	24.9±1.3	32.5

[a] Percent viability was determined 1 week after freezing.
[b] The freezing medium consisted of % DMSO, 40% FBS (heat-inactivated), and Mix ameba medium making the balance of 100% with a cell concentration of 1x10[6] amebae/ml.
[c] Greatest % viability.

Table 3. Viability of pathogenic free-living amebae frozen with various concentrations of FBS [a].

	Amebae			
serum[b]	*A. castellanii* (EI-212)	*N. australiensis* (PP-397)	*N. fowleri* (LEE)	Average of 3 species
0	18.6±8.7	6.7±3.7	15.0±4.0	13.4
10	22.1±6.2	69.3±3.9	35.9±4.4	42.4
20	39.1±10.1	72.0±2.6[c]	54.3±2.6[c]	55.1[c]
40	29.8±12.5	57.8±2.6	40.8±11.6	42.8
80	40.8±10.9	53.6±6.0	39.2±7.0	44.5
100	47.5±11.5[c]	0.9±0.9	2.4±1.0	16.9

[a] Percent viability was determined 1 week after freezing.
[b] The freezing medium consisted of 12% DMSO, % FBS (heat-inactivated), and Mix ameba medium making the balance of 100% with a cell concentration of 1x10[6] amebae/ml.
[c] Greatest % viability.

Table 4. Viability of pathogenic free-living amebae frozen with various bovine sera [a].

	Amebae			
serum[b]	*A. castellanii* (EI-212)	*N. australiensis* (PP-397)	*N. fowleri* (LEE)	Average of 3 species
Fetal bovine (not heat-inactived)	20.8±10.1	56.0±7.9	34.6±8.3	37.1
Fetal bovine (heat-inactived)	36.5±10.5	72.0±5.0	39.7±4.0	49.4
Bovine calf (not heat-inactived)	42.7±1.6[c]	80.4±7.6[c]	40.3±3.2	54.5
Bovine calf (heat-inactived)	36.2±1.9	78.7±8.0	68.5±4.1[c]	61.1[c]

[a] Percent viability was determined 1 week after freezing.
[b] The freezing medium consisted of 12% DMSO, 20% FBS bovine serum, and 68% Mix ameba medium with a cell concentration of 1x10[6] amebae/ml.
[c] Greatest % viability.

three species of amebae, producing an average of 41% protection (range 31–47%) (Table 1). Glycerol, FBS, HES, and PVP were able to afford protection to *A. castellanii* but not to *N. fowleri* or *N. australiensis*. Glycerol and DMSO are the cryoprotectants most commonly used in freezing protozoa. Hydroxyethyl starch and PVP have been used less frequently but have been shown to be effective cryoprotectants with certain organisms (Leef et al. 1979, Minjas and Townson 1980). The amount of DMSO that produced the greatest viability was 12% for *N. fowleri* and *N. australiensis* and 16% for *A. castellanii* (Table 2). Others have used 5% (White and Kilvington 1991, Simione and Daggett 1976) and 7.5% (Warhurst et al. 1980) DMSO for the cryopreservation of *N. fowleri*. *Entamoeba histolytica* has been frozen with 5% (Diamond et al. 1961), 10% (Gordon et al. 1969, Bosch and Frank 1972), and 15% (Diamond 1964) DMSO.

The protective action of serum in cryogenic preservation is well known. Mammalian cells are routinely frozen with FBS and DMSO with the amount of FBS ranging from 10–40% to as high as 90% (Hull 1983). In the present study we found that viability was greatest with 20% FBS for *N. fowleri* and *N. australiensis* and 100% for *A. castellanii* (Table 3). Additionally, we obtained greater viability with bovine calf serum than fetal bovine serum and with heat-inactivated than non-heat-inactivated serum (Table 4).

The cell concentration that resulted in the greatest viability was $1x10^6$ amebae/ml of freezing medium and averaged 67% for the three species (range 45–84%) (Table 5). The average viability for the three species was less at $1x10^5$ amebae/ml and least at $1x10^7$ amebae/ml. Cell concentration and the kind of cryoprotectant (Table 1) were the only two variables on which all three species of amebae exhibited complete agreement – DMSO was the best cryoprotectant and $1x10^6$ amebae/ml was the best cell concentration. Glucose has been used together with DMSO for freezing trypanosomes (Polge and Soltys 1960) and amebae (Diamond 1964). Dimethylsulfoxide is a rapidly penetrating protective agent and glucose is a slowly penetrating agent.

Table 5. Viability of pathogenic free-living amebae frozen at various cell concentrations [a].

	Amebae			
Cell concentration[b]	*A. castellanii* (EI-212)	*N. australiensis* (PP-397)	*N. fowleri* (LEE)	Average of 3 species
$1x10^5$	36.3±4.3	58.8±13.1	57.4±6.3	50.8
$1x10^6$	45.1±4.3[c]	83.9±3.0[c]	71.0±0.7[c]	66.7[c]
$1x10^7$	33.3±0.1	63.7±9.7	20.2±4.8	39.1

[a] Percent viability was determined 1 week after freezing.
[b] Cell concentration was amebae/ml of freezing medium which consisted of 12% DMSO, 20% BCS (heat-inactived), and 68% Mix ameba medium.
[c] Greatest % viability.

Table 6. Viability of pathogenic free-living amebae frozen with various concentrations of glucose [a].

	Amebae			
% glucose[b]	*A. castellanii* (EI-212)	*N. australiensis* (PP-397)	*N. fowleri* (LEE)	Average of 3 species
0	27.9±4.5	54.6±3.5	36.3±3.2	39.6
2	49.8±7.2	59.2±12.0	31.3±1.5	46.8
4	54.7±5.0[c]	59.6±6.2[c]	41.9±2.7	52.1[c]
8	37.9±1.8	29.1±9.4	51.3±2.6	39.4
10	40.1±8.5	29.8±8.3	56.2±3.6[c]	42.0
12	26.3±2.9	20.4±6.2	35.6±14.7	27.4
16	16.6±3.2	6.7±4.8	18.3±1.7	13.9

[a] Percent viability was determined 1 week after freezing.
[b] Glucose was added to the freezing medium to give the desired concentration. Freezing medium consisted of 12% DMSO, 20% BCS (heat-inactived), and 68% Mix ameba medium with a cell concentration of $1x10^6$ amebae/ml.
[c] Greatest % viability.

Table 7. Viability of pathogenic free-living amebae using various freezing schedules [a].

	Amebae			
Freezing schedule[b]	*A. castellanii* (EI-212)	*N. australiensis* (PP-397)	*N. fowleri* (LEE)	Average of 3 species
A	13.2±3.5	42.2±5.9	11.3±2.4	22.2
B	23.4±9.6	75.2±1.0[c]	29.1±4.4	42.6
C	27.2±3.4[c]	73.3±0.9	30.2±7.4[c]	43.6[c]
D	7.1±1.3	52.1±3.9	18.4±8.0	25.9

[a] Percent viability was determined 1 week after freezing.
[b] Freezing schedules were:

	A	B	C	D
30 min at 23° C			X	X
60 min at –20° C		X	X	
Directly to –70° C	X	X	X	X

Freezing medium was 12% DMSO, 20% BCS (heat-inactivated), and 68% Mix ameba medium with a cell concentration of $1x10^6$ amebae/ml.
[c] Greatest % viability.

Table 8. Viability of pathogenic free-living amebae frozen at various phases of growth [a].

	Amebae			
Phase of growth[b]	*A. castellanii* (EI-212)	*N. australiensis* (PP-397)	*N. fowleri* (LEE)	Average of 3 species
Exponential	42.9±9.7[c]	73.3±3.9[c]	32.2±3.5	49.5[c]
Stationary	29.3±4.4	52.5±1.9	41.2±0.5[c]	41.0
Decline	25.7±1.6	17.9±0.8	34.7±3.2	26.1

[a] Percent viability was determined 1 week after freezing.
[b] Freezing medium was 12% DMSO, 20% BCS (heat-inactived), and 68% Mix ameba medium with a cell concentration of $1x10^6$ amebae/ml.
[c] Greatest % viability.

Diamond (1964) found that glucose enhanced the protective effect of DMSO in freezing *Entamoeba invadens*, a pathogenic ameba of reptiles. Our results concur (Table 6). Greatest viability occurred with 4% glucose for *A. castellanii* and *N. australiensis* and 10% glucose for *N. fowleri*. An increase in glucose beyond the optimum resulted in decreased viability. For routine freezing of pathogenic free-living amebae we prepare the freezing medium by adding glucose to Mix ameba medium so that the final concentration is 4%.

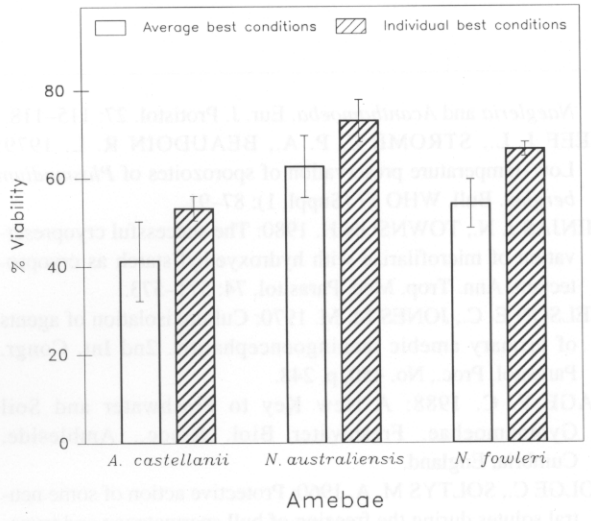

Fig. 1. Viability of pathogenic free-living amebae frozen using average best conditions for the 3 species and individual best conditions. Freezing conditions are those listed in Tables 1–8, except with *A. castelanii* (EI-212) 80% serum was used rather then 100% (Table 3). Percent viability was determined 1 week after freezing.

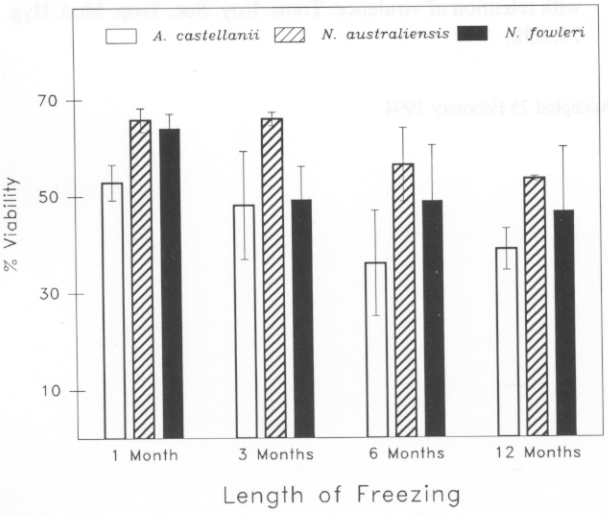

Fig. 2. Viability of pathogenic free-living amebae frozen for 12 months. Freezing conditions were average best conditions: $1x10^6$ exponentialy drowing amebae/ml freezing medium consisting of 12% DMSO, 20% heat-inactived bovine calf serum, 4% glucose, in Mix ameba medium; 30 min equilibration at 23° C followed by 60 min at –20° C and storage at –70° C.

The freezing schedule that resulted in the greatest viability was one in which amebae were mixed with the freezing medium, placed in cryovials, and allowed to equilibrate at 23° C (room temperature) for 30 min. Cryovials were transferred to –20° C for 60 min and then stored at –70° C (Table 7). Viability was least when amebae were transferred directly to –70° C after mixing in the freezing medium. The second lowest average viability was for amebae equilibrated at room temperature (23° C) and then transferred to –70° C. Viability was greatly enhanced by storage at –20° C for 60 min before transfer to –70° C. Prior equilibration at room temperature only slightly increased viability. Others have also reported the efficacy of stepwise freezing of *N. fowleri*. Kilvington and White (1991) used –20° C for 60 min, followed by –70° C for 60 min, and storage at –196° C (liquid nitrogen). Simione and Daggett (1976) used room temperature for 30 min, followed by –55° C for 60 min, and storage at –155° C (vapor phase of liquid nitrogen). Warhurst et al. (1980) used 23° C for 15 min, followed by –50° C for approximately 75 min, and storage at –196° C.

The average best viability was achieved with cells that were frozen during exponential growth (Table 8), followed by amebae in stationary-growth phase and finally decline-phase amebae. Viability was greater for amebae frozen in a medium that was a composite of their individual best conditions (Tables 1–8) as compared to the freezing medium that represented the average best conditions for the three species combined (Fig. 1). Nonetheless, the average viability was 53% for the three species (range 41–63%) for the average best freezing medium.

Fig. 2 illustrates the percent viabilities of the three species at specific intervals during 12 months of freezing. Although viability decreases with time, it appears that the greatest decrease occurred during the first 6 months of freezing. The overall reduction in viability at 12 months of freezing was 19% for *N. australiensis* and 27% for *N. fowleri* and *A. castellanii*. Viability at 1 year of freezing was 39% for *A. castellanii*, 47% for *N. fowleri*, and 53% for *N. australiensis*. Using the greatest percent viabilities from each of the eight tables and the two figures, the overall average viability for the three species was 45% for *A. castellanii* (range 27–54%), 54% for *N. fowleri* (range 30–71%), and 66% for *N. australiensis* (range 31–84%). Others have reported 8% viability (Kilvington and White 1991) and 63% intact cells (Simione and Daggett 1976) for *N. fowleri* frozen for 14 days and 7 days, respectively.

In summary, the average conditions that were optimal for the cryopreservation of the three species of pathogenic free-living amebae studied are: $1x10^6$ exponentially growing amebae/ml of freezing medium consisting of 12% DMSO, 20% heat-inactivated bovine calf serum, 4% glucose, in Mix ameba medium; 30 min equilibration at 23° C (room temperature), followed by 60 min at –20° C, with storage at –70° C. Under these conditions viability was 53% for *A. castellanii*, 64% for *N. fowleri*, and 66% for *N. australiensis* after 1 month of freezing (Fig. 2).

Acknowledgments. We thank Susan Brooks for typing the manuscript and preparing the figures and Steven Phillips for assistance with the SigmaPlot program. This investigation was supported by grants R-814327 and R-818106 from the United States Environmental Protection Agency.

REFERENCES

ANONYMOUS 1986: Freezing and thawing serum and cells. Art to Science, Newsletter of HyClone Laboratories, Inc., Logan, Utah 5: 1–6.

BALAMUTH W. 1964: Nutritional studies on axenic cultures of *Naegleria gruberi*. J. Protozool. 11 (Suppl.): 19–20.

BOSCH I., FRANK W. 1972: Beitrag zur Gefrierkonservierung pathogener Amoeben und Trichomonaden. Z. Parasitenkd. 38: 303–312.

DIAMOND L. S. 1964: Freeze–preservation of protozoa. Cryobiology 1: 95–102.

DIAMOND L. S., MERYMAN H. T., KAFIG E. 1961: Storage of frozen *Entamoeba histolytica* in liquid nitrogen. J. Parasitol. 47 (Suppl.): 28–29.

GORDON R. M., GRAEDEL S. K., STUCKI W. P. 1969: Cryopreservation of viable axenic *Entamoeba histolytica*. J. Parasitol. 55: 1087–1088.

HULL R. N. 1983: Successful recovery of cells following long term storage in liquid nitrogen. American Type Culture Collection 3: 1.

JOHN D. T. 1993: Opportunistically pathogenic free–living amebae. In: J. P. Kreier, J. R. Baker (Eds.), Parasitic Protozoa, 2nd Ed. Academic Press, Inc., San Diego, California, 3pp.: 143–246.

KILVINGTON S., WHITE D. 1991: A simple method for the cryopreservation of free–living amoebae belonging to the genera *Naegleria* and *Acanthamoeba*. Eur. J. Protistol. 27: 115–118.

LEEF J. L., STROME C. P. A., BEAUDOIN R. L. 1979: Low–temperature preservation of sporozoites of *Plasmodium berghei*. Bull. WHO 57 (Suppl. 1): 87–91.

MINJAS J. N., TOWNSON H. 1980: The successful cryopreservation of microfilariae with hydroxyethyl starch as cryoprotectant. Ann. Trop. Med. Parasitol. 74: 571–573.

NELSON E. C., JONES M. M. 1970: Culture isolation of agents of primary amebic meningoencephalitis. 2nd Int. Congr. Parasitol. Proc., No. 450, p. 248.

PAGE F. C. 1988: A New Key to Freshwater and Soil Gymnamoebae. Freshwater Biol. Assoc., Ambleside, Cumbria, England.

POLGE C., SOLTYS M. A. 1960: Protective action of some neutral solutes during the freezing of bull spermatozoa and trypanosomes. In: A. S. Parkes, A. U. Smith (Eds.), Recent Research in Freezing and Drying, Blackwell Scientific Publications, Oxford, England, pp. 87–100.

SIMIONE F. P., Jr., DAGGETT P.–M. 1976: Freeze preservation of pathogenic and nonpathogenic *Naegleria* species. J. Parasitol. 62: 49.

WARHURST D. C., CARMAN J. A., MANN P. G. 1980: Survival of *Naegleria fowleri* cysts at 4°C for eight months with retention of virulence. Trans. Roy. Soc. Trop. Med. Hyg. 74: 832.

Received 16 November 1993

Accepted 25 February 1994