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The maintenance of free-living amoebae by cryopreservation

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Abstract: We have successfully cryopreserved free-living amoebae in order to maintain them feasibly under the conditions in our laboratory. The viability of trophozoites was higher when frozen by slow cooling (overall 0.7°C/min) than by fast cooling (overall 1.3°C/min). Glycerol and dimethylsulfoxide at the final concentration of 7.5% each was used for cryopreservation of free-living amoebae trophozoites. The survival rate was 2~39% after storage in the liquid nitrogen for 60 days. Gross cultural or morphological changes were not noted in trophozoites thawed from frozen suspensions.

Key words: Cryopreservation, free-living amoebae, survival rate, liquid nitrogen

Conventional methods for preserving parasites involve *in vitro* culture or animal passage. These are expensive, tedious and so risky—the organisms are often prone to genetic drift or selection and vulnerable to loss through human error or contamination. When the organisms are stored by cryopreservation, all of these are considerably reduced (James, 1988). There have been some reports regarding such issues as the practicality and efficiency of re-establishing a functional full-scale life cycle operation from cryopreserved organisms (Cohen and Eveland, 1984; Cooper *et al.*, 1989) and a lot of reports relating to advanced cryopreservation techniques of many protozoan (Lumsden *et al.*, 1966; Morii *et al.*, 1988) or helminthic species (James, 1985; Rossi and Pozio, 1988). A variety of cryopreserving methods now have been adopted for many species of amoebae (Dwyer and Honigberg, 1971; Raether and Uphoff, 1976). Simione and Daggett (1976) reported the successful cryopreservation of two *Naegleria* species. But the cryopreservation of *Acanthamoeba* might not be reported.

We have attempted maintaining free-living amoebae by cryopreservation that had been preserved by a successive batch culture in our laboratory. The amoebae used were *Acanthamoeba culbertsoni, A. polyphaga, Naegleria fowleri* and *N. gruberi*. These were received from Jardin, J.B.-Prince Leopold Institute of Tropical Medicine, Belgium. The amoebae were cultivated in CGV (Casitone-Glucose-Vitamine) medium for *Acanthamoeba* and CGVS (Casitone-Glucose-Vitamine-Serum) medium for *Naegleria* (Willaert, 1971). *A. culbertsoni* and *N. fowleri* were cultured in 37°C incubator (NaPCO, Portland, Oregon), *A. polyphaga* and *N. gruberi* in 25°C incubator (Samwha, Seoul, Korea). The cultivated amoebae suspensions were collected in 50-ml centrifuge tube, and centrifuged at 1,000 rpm for 10min. The amoebae were resuspended and counted. The cryoprotector, prepared in the respective medium, was added to give a final concentration of 7.5% (v/v) and a final prefreeze count of 2.5×10^6 organisms/ml. The suspension was then dispensed into sterile screw-capped 1.2-ml ampoules in 1ml aliquots, and allowed to

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remain at room temperature for 30 min. The ampoules were placed in a box made of polystyrene and cooled by means of two methods. Fast cooling was performed by the method of Simione and Daggett(1976), and slow cooling by the method of Neal *et al.* (1974). The cooled ampoules were plunged into liquid nitrogen(Fig. 1). Recovery of viable organisms was accomplished by rapid thawing of the ampoule and contents by immersion in a 37°C water bath. When thawed, the suspension of organisms was inoculated directly into a suitable culture medium, and the number of viable organisms was determined. In tests of viability, when amoebae

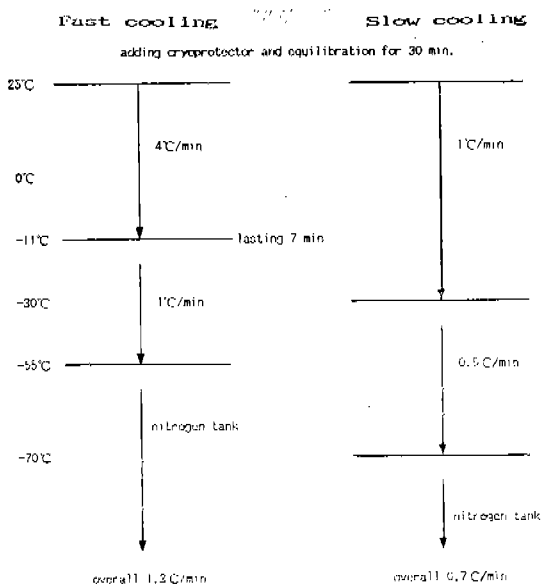

Fig. 1. The diagram of cooling rate.

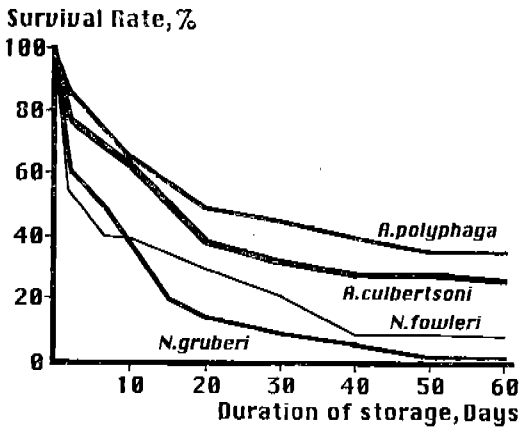

Fig. 2. The recoveries after storage in the liquid nitrogen. The initial suspension of amoebae contained 2×10^5 trophozoites/ampoule.

were observed growing in the primary inoculation and the growth was vigorous, the method of cryopreservation was determined to be successful. If the growth was poor, a subculture was made to see if growth was improved. Differences in the viability of the amoebae recovered were observed and cryopreservation using slow cooling was found to be more effective (Table 1). In view of these observations, testing over the length of time of storage was carried out using slow cooling cryopreservation. The maximum length of time that amoebae will survive in the liquid nitrogen has not yet been observed. The summary of data shown in Fig. 2 represents successful recoveries that have been made from materials stored for up to 60 days. It is clear

Table 1. The comparison of survival rates (%) in the case of 2.5×10^6 of *Naegleria fowleri* and *Acanthamoeba culbertsoni* trophozoites by means of fast cooling and slow cooling

Amoeba	Time of storage (days)	7.5% Glycerol		7.5% Dimethyl sulfoxide	
		Fast cooling	Slow cooling	Fast cooling	Slow cooling
Naegleria fowleri	10	2×10^5(8)*	5×10^5(20)	5×10^4(2)	1.3×10^6(52)
	20	1.3×10^5(5.2)	3.7×10^5(14.8)	2.1×10^4(0.84)	8.5×10^5(34)
	30	1×10^5(4)	2.1×10^5(8.4)	1×10^4(0.4)	5×10^5(20)
Acanthamoeba culbertsoni	10	6.6×10^5(26.4)	1.7×10^6(68)	2.4×10^5(9.6)	1.6×10^6(64)
	20	3.8×10^5(15.2)	7.6×10^5(30.4)	8×10^4(3.2)	1×10^6(40)
	30	3×10^5(12)	5.4×10^5(21.6)	7×10^4(2.8)	8×10^5(32)

* Percentage of recovery.

that the period of storage was not long in this study, and could be prolonged by reason that the survival rate was comparatively high (2～39%) after storage in liquid nitrogen for 60 days, and no gross cultural or morphological changes were noted in amoebae derived from frozen suspensions. Although, the data were not shown, the recovery rate of amoebae was low, being 0.5～20% after 6 months. But we prepared for freezing a suspension containing 2×10^6 amoebae/ml. Therefore even with a recovery of 0.5% the number of normal amoebae inoculated into the seeded culture medium is 10^4 amoebae. Thus it seems possible that free-living amoebae could have been maintained by freezing preservation for 6 months.

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=국문요약=

자유생활 아메바의 냉동보관

연세대학교 의과대학 기생충학교실 및 열대의학연구소

서 성 아·용 태 순·임 경 일

자유생활 아메바를 효과적으로 유지할 목적으로 냉동보관을 시도하였다. Glycerol과 Dimethylsulfoxide를 각각 최종농도 7.5%가 되도록 아메바현탁액에 첨가하였다. 아메바 영양형을 냉동하는 과정에서 온도 하강속도가 빠를때(평균 1.3°C/min)보다 느릴때 (평균 0.7°C/min) 생존력이 더 높았다. 액체질소 속에서 60일동안 보관한 후의 생존율은 처음 넣어준 아메바 영양형 수의 2～39%에 달하였다. 냉동 보관했던 아메바를 해동했을 때 어떤 형태적 변화나 배양했을 때의 문제점도 관찰되지 않았다.

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