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On the Cryo-biological Study of the Parasitic Protozoa (2) The low temperature preservation in freezers

Akira MIYATA

Department of Epidemiology, Institute for Tropical Medicine, Nagasaki University

(Chief : Prof. Toshio NAKABAYASHI)

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Abstract

The following results were obtained :

1. *Entamoeba histolytica* survived for only several days in a sample with 5% dimethyl sulfoxide at −75C after pre-cooling in a −25C freezer.

2. In a sample with 7.5% glycerol stored at −20C, RH strain of *Toxoplasma gondii* survived for at least 75 days, and *Plasmodium berghei* was preserved for 49 days, whereas *Trypanosoma gambiense* survived for only 3 days.

3. *T. gambiense*, *P. berghei*, and RH strain of *T. gondii* could be easily stored at −75C in the presence of 7.5% glycerol, and even without any cryo-protective substance, the latter two species survived for about 80 days.

4. In spite of several trials, Beverley strain of *T. gondii* could not survive at low temperatures except only one sample stored at −20C for one day had an infectivity to a mouse.

5. The most interesting finding was that the survival period of protozoa in the sample prepared with inactivated bovine serum was somewhat longer than that prepared with physiological saline.

Introduction

It was often reported that various kinds of parasitic protozoa might survive for a long period at an ultra-low temperature lower than −75C in the presence of glycerol or dimethyl sulfoxide. The author also reported his results of the preservation of trichomonads in freezers at −25C and −75C (Miyata, 1973), and the present paper contains the results obtained from several parasitic protozoa other than trichomonads.

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Materials and Methods

The following species and strains of parasitic protozoa were used:

Entamoeba histolytica, Laredo strain (supplied by Prof. K. Asami, Keio University)

Trypanosoma gambiense (supplied by Prof. S. Inoki, Osaka University)

Plasmodium berghei (supplied by Prof. Q. M. Geiman, Stanford University School of Medicine)

Toxoplasma gondii, RH and Beverley strains (supplied by Prof. S. Takada, Osaka City University)

E. histolytica was cultivated in Tanabe-Chiba Medium (1928) which consisted of 10 g of agar and 1 g of sodium asparate in 1 liter of Ringer's solution. Each 5~6 ml of the solution was pippetted into a test tube, and sterilized 3 times intermittently by 100C steam for 30 minutes, then cooled in the state of reclining at an angle of 5~6° at room temperature. Just before use, 5ml of Ringer's solution containing 5% inactivated bovine serum and 1 or 2 loops of sterilized rice powder were added into the test tube. The other protozoa were maintained by serial mouse-passages.

Dimethyl sulfoxide (DMSO) or glycerol (GLY) was used as the cryo-protective substance. Twice of the required final concentration (v/v) of a cryo-protective substance was diluted with an equal volume of a protozoan suspension prepared in distilled water or physiological saline.

One ml of the sample was distributed into a small test tube or an ampoule, then closed by a rubber cap or sealed by heat, respectively.

The samples were allowed to equilibrate for 10 minutes with DMSO or 30 minutes with GLY, then stored in freezers adjusted at −20C, −25C, or −75C. The cooling speed was checked for each experiment with an electric thermocouple.

For examinations, the samples were taken out from the freezer at various intervals and thawed rapidly in a 37C water bath.

Results

(1) *Entamoeba histolytica*

One week cultured *E. histolytica* was gathered from the bottom of the media, and used for the experiment. At first the sample with or without 5% DMSO was cooled directly in a −75C freezer, but after the temperature of the sample reached −30C, no survival amoeba was detected. Then, in the next attempt, the pre-cooling method reported in the previous paper (Miyata, 1973) was used; The samples with 5% DMSO were cooled in a −25C freezer for 1.5 hours, then a half of the pre-cooled samples was transfered into the −75C freezer and the rest stored still in the −25C freezer (Table 1). The surviving amoebae were detected microscopically in the sample of the 6-day storage at −25C. The similar results were obtained in the −75C freezer. The longest survival record of *E. histolytica* at −75C was only 4 days in the table 1,

Table 1. Freezing preservation of *Entamoeba histolytica* in the presence of 5% DMSO

Storage Period in −25C Freezer		Storage Period in −75C Freezer after Precooling in −25C Freezer	
at 0C[1]	+	1 hour[3]	+
at super-cooled state	+	1 day	+(+)
at −20C	+	2 days	+(+)
1 hour[2]	+	4 days	−(+)
1 day	+(+)[4]	10 days	−
2 days	+	16 days	−(−)
4 days	−(+)		
6 days	+		
10 days	−		
14 days	−(−)		

Notes: 1) Sample was examined at 0C, and motile amoebae were detected microscopically as shown as +.
2) After the temperature of a sample reached −20C, the sample was stored for one hour.
3) When the temperature of a sample reached −20C, all samples were stored for one hour in the −25C freezer, then half of the samples were transfered into the −75C freezer.
4) After microscopic examination, the sample was inoculated a fresh medium and cultivated for one week in a 37C incubator and the result was shown inside of ( ).

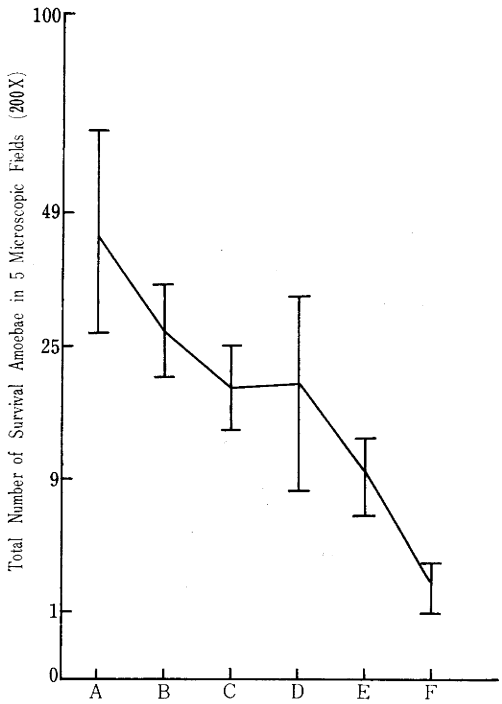

Fig. 1. Decrease of *Entamoeba histolytica* in the −25C freezer.
In the following 6 points, the average and the range of the survival number in 5 microscopic fields were shown in each results.
A: at room temperature B: at 0C
C: at super-cooled state D: at −20C
E: sample stored for one hour after temperature reached −20C
F: sample stored for 2 days after temperature reached −20C

and the record was prolonged to 8 days in the repeated trials. Various concentrations of GLY and DMSO were also examined in succeeding experiments, and no successful result was obtained.

In the −25C freezer, the decrease in the number of motile amoebae was proportional to the drop of the sample's temperature, (Fig. 1), indicating that longer pre-cooling in the freezer was injurious to the survival of the amoeba.

(2) *Trypanosoma gambiense*

A total of 8 ml blood was collected by the heart-puncture with heparinized syringes from ten mice infected by *T. gambiense*, and added to 22 ml of inactivated bovine serum. The mixture was divided into 3 parts as follows:

Group 1. 10 ml of the mixture + 10 ml of 15% GLY prepared in distilled water (The final concentration of GLY was 7.5%.)

Group 2. 10 ml of the mixture + 10 ml of physiological saline

Group 3. 10 ml of the mixture + 10 ml of inactivated bovine serum

Each one ml was distributed into 20 small test tubes, and half of them were placed in a −20C freezer, the rest in a −75C freezer. Before cooling, the final average number of trypanosomes in the sample was 40,000 per mm^3. Survivals at various periods of the storage were shown in Table 2. In the −20C freezer, trypanosomes could survive for at least 3 days in the sample with GLY (Group 1), whereas in the other two groups no survival was detected. In the −75C freezer, surviving trypanosomes were found after the storage for 80 days in Group 1 and

Table 2. Freezing preservation of *Trypanosoma gambiense*

Period Stored (days)	Group of Sample	Stored in −20C Freezer		Stored in −75C Freezer	
		Microscopic Examination	Mouse Inoculation	Microscopic Examination	Mouse Inoculation
1	1	+	4 D[1]	##	4 D
	2	−	S[2]	#	4 D
	3	−	S	−	5 D
3	1	##	7 D	##	4 D
	2	−	S	#	3 D
	3	−	S	−	5 D
7	1	−	S	#	3 D
	2	−	S	#	3 D
	3	−	S	−	5 D
14	1			##	3 D
	2			+	4 D
	3			−	5 D
31	1			##	3 D
	2			+	5 D
	3			−	5 D
50	1			##	3 D
	2			−	9 D
	3			−	5 D
80	1			##	8 D
	2			−	S
	3			−	9 D

Notes : A sample of each group consists as follows:
Group 1. 0.5ml of the mixture with trypanosomes + 0.5ml of 15% GLY
Group 2. 0.5ml of the mixture with trypanosomes + 0.5ml of physiological saline
Group 3. 0.5ml of the mixture with trypanosomes + 0.5ml of inactivated bovine serum
1) Four days later, the inoculated mouse died of the heavy infection of *T. gambiense*.
2) The inoculated mouse survived for longer than 10 days without infection.
: Many survival trypanosomes were detected from the sample (more than 50 survivals per one microscopic field of 400×, and average survival rate of trypanosomes was 10~20%).
: Less than 50 survival trypanosomes were detected in each microscopic field and the average survival rate was lower than 10%.
+ : Very few survival trypanosomes were detected in the sample.
− : Survival trypanosome was never detected microscopically in the sample.

3. In Group 2, however, the number of motile trypanosomes decreased proportionally to the storage period, and at last the sample stored for 80 days did not infect the mice. It might be a most interesting finding that any motile trypanosome was never observed microscopically in all the samples of Group 3 stored

Table 3. Freezing preservation of *Plasmodium berghei*

Period Stored (days)	Group of Sample	Stored in −20C Freezer		Stored in −75C Freezer	
		Blood Smear	Survival Days	Blood Smear	Survival Days
1	1	4[1]	11[2]	4	8
	2	10	S	4	8
	3	10	26	4	21
3	1	4	9	4	20
	2	Neg[3]	S[4]	4	16
	3	8	19	4	22
7	1	4	13	4	16
	2	Neg	S	4	16
	3	9	21	4	16
14	1	5	13	5	16
	2	Neg	S	5	18
	3	Neg	S	5	12
28	1	7	14	4	19
	2	Neg	S	4	23
	3	Neg	S	4	18
35	1	6	19		
	2				
	3	Neg	S		
41	1	7	20	4	11
	2			4	16
	3	Neg	S	4	11
49	1	7	16		
	2				
	3	Neg	S		
56	1	Neg	S	5	9
	2			5	13
	3			5	16
62	1	Neg	S	7	23
	2			7	15
	3			7	16
78	1			4	16
	2			4	20
	3			4	12

Notes : A sample of each group consist as follows:
Group 1. 0.5ml of the mixture with parasite + 0.5ml of 15% GLY
Group 2. 0.5ml of the mixture with parasite + 0.5ml of physiological saline
Group 3. 0.5ml of the mixture with parasite + 0.5ml of inactivated bovine serum

1) Four days later malaria parasites were detected from the blood smear of the inoculated mouse.
2) The mouse died of malaria at 11 days after the inoculation.
3) Malaria parasite was never detected from the inoculated mouse during an observation period of a month.
4) The mouse survived for a month after the inoculation.

at −75C during the preservation period, although mice inoculated with the samples were killed by heavy trypanosome infection.

(3) *Plasmodium berghei*

A total of 7 ml blood was collected by the heart-puncture with heparinized syringes from ten mice infected with *P. berghei*, and mixed with 23 ml of inactivated bovine serum. A total of 30 ml mixture was divided into 3 groups as follows:

Group 1. 10 ml of the mixture + 10 ml of 15% GLY in distilled water (The final concentration of GLY was 7.5%.)
Group 2. 10 ml of the mixture + 10 ml of physiological saline
Group 3. 10 ml of the mixture + 10 ml of inactivated bovine serum

Each one ml of the sample was distributed into 20 small test tubes. In each group, 10 samples were placed in a −20C freezer, and the rest in a −75C freezer. It was difficult to detect the surviving malaria parasite microscopically without staining, because this protozoa is an intracellular parasite of red blood cell. Each 0.3 ml of sample was inoculated intraperitonealy to three fresh mice. Table 3 presents the days from the inoculation until the malaria parasite could be detected from the blood and until the mouse was killed by malaria. In the −20C freezer, the sample of Group 1 stored for 49 days could infect the mouse, although the samples of Group 2 stored for 3 days or of Group 3 for 14 days could not. According to the microscopic examination of all the samples preserved at −20C, few red blood cells were found from Group 1, but most of the red blood cells were destroyed in the other groups. In the −75C freezer, the samples of all three groups could infect those mice during the whole preservation period, and a lot of red blood cells were detected microscopically from the sample of Group 1, but in the other groups few of the cells were observed.

(4) *Toxoplasma gondii*

(a) RH strain

RH strain which is a virulent strain isolated from a patient, killed a mouse within 4~5 days after inoculation, and many toxoplasmas were detected from the whole body of the mouse, especially ascites from 0.8 to 1.2 ml. A total of 10 ml ascites from 10 mice infected with the strain were mixed in 30 ml of physiological saline, and divided into 2 groups as follows:

Group 1. 20 ml of the mixture + 20 ml of 15% GLY in distilled water (The final concentration of GLY was 7.5%.)
Group 2. 20 ml of the mixture + 20 ml of physiological saline

Each one ml was distributed into 40 ampoules and sealed by heat, then half of the samples were stored in a −20C freezer and the rest in a −75C freezer. After thawing in a 37C water bath, each 0.3 ml of the sample was inoculated intraperitonealy to 3 fresh mice and the survival days of the mice after the inoculation were shown in Table 4. Although the table contains data for 30-day storage, an experiment repeated under the same conditions clearly showed that RH strain

could be stored safely for at least 90 days at −75C in the presence or absence of GLY or DMSO. The survival days of the mice inoculated with the preserved sample were prolonged 2 or 3 days in comparison with the mouse inoculated with a fresh sample of the same batch, but it was not clear whether the virulence of the strain had changed or living protozoa had decreased in number during the preservation period. Because dead toxoplasmas were not destroyed by the freezing and thawing procedure, it was difficult to distinguish the living individuals from the dead ones by means of microscopic observation.

(b) Beverley strain

The mouse inoculated with Beverley strain could survive, with a healthy appearence, for one or two months, but about one month later a number of cysts could be detected microscopically from the brain. After one month from the inoculation with the strain, 3 brains of the mice were ground in a motor with 10 ml of physiological saline. The suspension was filtered with double layers of gauze, then 20 ml of physiological saline were added and divided into 3 groups as follows:

Group 1. 10 ml of the mixture + 10 ml of 15% GLY in distilled water (The final concentration of GLY was 7.5%.)

Group 2. 10 ml of the mixture + 10 ml of 7.5% GLY in distilled water (The final concentration of GLY was 3.8%.)

Group 3. 10 ml of the mixture + 10 ml of physiological saline

Table 4. Freezing preservation of *Toxoplasma gondii* (RH strain)

Period Stored (days)	Group of Sample	Stored in −20C Freezer	Stored in −75C Freezer
Control (0 day)[1]	1 2	5, 5, 6 5, 6, 7	
1	1 2	6, 7, 7 10,10,10	8, 8, 8 8, 8, 8
10	1 2	8, 8, 8 S, S, S[2]	
16	1 2	7, 9, 9 S, S, S	7, 7, 7 8, 8, 9
21	1 2	7, 7,10	
30	1 2	8, 8, 8	8, 9, 9 9, 9,10[3]
35	1 2	7, 8, 8	
40	1 2	7, 7, 8	
45	1 2	8, 9,13	
50	1 2	10,14	
63	1 2	8, 9,10	
70	1 2	9, 9, 9	
75	1 2	8, 8, 9	

Notes : A sample of each group consists as follows:
Group 1. 0.5 ml of the mixture with parasite + 0.5ml of 15% GLY
Group 2. 0.5 ml of the mixture with parasite + 0.5 ml of physiological saline
1) The equal volume of the sample was inoculated into 3 mice intraperitoneally just before cooling, and the mice died of heavy infection at 5, 6, and 7 days later.
2) The inoculated mouse survived for longer than a month, then the mouse was examined, but toxoplasma was not detected.
3) The −75C freezer became out of order 30 days after than start of the experiment, then all samples were warmed naturally up to a room temperature, and no survival toxoplasma was detected.

Each one ml was distributed into 20 small test tubes, and half of the samples were stored in a −25C freezer and the rest in a −75C freezer. After thawing, each 0.4 ml of a sample was inoculated into 2 fresh mice, and 6 weeks later the brains were examined microscopically. The cysts of the strain were detected only from the brain of one mouse inoculated with a sample in Group 2 of one-day storage in the −25C freezer, and all the mice inoculated with other samples were negative (Table 5). According to the microscopic examination of these negative samples before the inoculation, a few of the cysts was detected without damage, but they already lost the infectivity to the mouse.

Table 5. Freezing preservation of *Toxoplasma gondii* (Beverley strain)

Period Stored (days)	Group of Sample	Stored in −20C Freezer		Stored in −75C Freezer	
		Microscopic Examination[1]	Mouse Inoculation[2]	Microscopic Examination	Mouse Inoculation
1	1	Neg	##	+	Neg
	2	+	Neg	Neg	Neg
	3	Neg	Neg	Neg	Neg
7	1	Neg	Neg	Neg	Neg
	2	Neg	Neg	Neg	Neg
	3	Neg	Neg	Neg	Neg

Notes : A sample of each group consists as follows:
Group 1. 0.5 ml of the mixture with cysts + 0.5 ml of 15% GLY
Group 2. 0.5 ml of the mixture with cysts + 0.5 ml of 7.5% GLY
Group 3. 0.5 ml of the mixture with cysts + 0.5 ml of physiological saline
1) The sample was examined microscopically after warming, and if cyst was detected, it was shown as +.
2) The sample was inoculated into a fresh mouse after microscopic examination, and one month later the mouse was examined microscopically and if cyst was detected from the brain, it was shown as cyst +.

Discussion

In the presence of glycerol at −20C, RH strain of *Toxoplasma gondii* were stored safely for at least 75 days and *Plasmodium berghei* survived for 49 days, but the longest survival record of *Trypanosoma gambiense* was only 3 days. It seems that the preservation of those three species at −75C is rather easy, and, even without any cryo-protective substance, samples of the two species except *T. gambiense* have survived for at least 80 days. According to a short report by Weinman (1958), *Trypanosoma rhodesiense* survived for 8 years at −70 C, and it might be longest record so far in the low temperature preservation of the parasitic protozoa. If the temperature of the −75C freezer could be kept constant during a preservation period, it might be possible to store for several years not only trypanosome but also malaria parasite and RH strain of toxoplas-

ma. The most important matter in the presrevation was how to control the fluctuation of the temperature at least within 5~10C.

The most interesting finding was that the survival period of protozoa in the sample prepared in inactivated bovine serum was somewhat longer than that prepared in physiological saline. It might be likely that the serum has some cryoprotective action to protozoa cells in the low temperature preservation.

In Beverley strain of *T. gondii*, only one sample of one-day storage at −25C infected the inoculated mouse, but the other samples did not infect. This fact might be explained by the assumption that the cyst wall of the strain was very hard and even if ice was formed in the cyst, trophozoites in the cyst were destroyed by the ice mechanically, and the cyst was also broken by the ice. According to Nakabayashi *et al.* (1967), the cysts of the strain survived for 67 days in a low temperature room adjusted 0 ± 2C, thus it seems that the ice formation was very injurious to the cyst.

The preservation of *Entamoeba histolytica* was not successful in the present experiments, because the amoebae survived only for several days at −75C even in the presence of GLY or DMSO, although Fulton *et al.* (1953) succeeded to detect survival amoebae from the cultures inoculated with samples that contained 5 or 10% GLY after 65−day storage at −79C; and some other researchers also reported their successes concerning the low temperature preservation of *E. histolytica* (Diamond *et al.*, 1963, Gordon *et al.*, 1969, and Kasprzak *et al.*, 1970). The attempt to preserve amoeba is being continued by the present author.

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寄生性原虫類の低温生物学的研究

2. フリーザーによる低温保存

宮　田　　彬

長崎大学熱帯医学研究所疫学部
(主任：中林敏夫教授)

摘　　要

本報では，先に報告（宮田，1973）したトリコモナス類以外の寄生性原虫類について，−20度，−25度，−75度の3種のフリーザーを用いて，凍結保存を試みた．得られた成績は，次の通りである．

1．赤痢アメーバは，凍結保存が困難で，−25度フリーザー中で1時間半予備凍結後，−75度フリーザーへ移し保存しても，せいぜい数日間保存できたにすぎない．

2．7.5%グリセリンを加えた試料を，−20度のフリーザー中に凍結保存する場合は，トキソプラズマRH株は，少なくとも75日，ネズミ・マラリアは，49日保存できたが，ガンビエ・トリパノゾーマは，3日間しか保存できなかった．

3．上記3種の原虫は，7.5%グリセリンを加え，−75度フリーザー中で凍結した場合は，大変長期間保存することができる．またトリパノゾーマを除く2種の原虫は，例え凍害保護剤を加えなくても，およそ80日間保存できた．

4．何回も試みたが，トキソプラズマのビバリー株の保存は難しく，−20度で凍結した7.5%グリセリン加試料中の1本だけが1日保存後もマウスに感染力をもっていた．しかしその他の試みは全て失敗に終った．

5．原虫を非働化牛血清中で凍結する場合は，生理的食塩水中で凍結するよりも，原虫の保存期間が長くなった．