Toxoplasma gondii from liquid nitrogen for continuous cell culture: methods to maximise efficient retrieval

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ABSTRACT

This study aims to increase the efficiency of continuous growth of *Toxoplasma gondii* in HeLa cells from tachyzoite stocks frozen in liquid nitrogen. Freezing and retrieval of tachyzoites for continuous cell culture requires more stringent protocols than those published for animal culture. The freezing and retrieval conditions are optimised so that a quality harvest ($\geq 1 \times 10^6$ tachyzoites/mL, $\geq 90\%$ viability) can be produced using *T. gondii* recovered from liquid nitrogen as fast and reliably as possible. Retrieval success rate increased from 36% to 100%. An improved freezing procedure using chilled reagents and freshly harvested parasites, and adoption of an effective recovery protocol with retrieval of 3×10^7 tachyzoites into 75cm^2 flasks, change of maintenance media after six hours and subsequent blind passage all contributed to this success. The result is faster and more dependable production of *T. gondii* for diagnostic and experimental use.

KEY WORDS: Cell culture. Liquid nitrogen. Toxoplasma.

Introduction

Toxoplasma gondii has been routinely cultured for many years to provide live tachyzoites for use in diagnostic tests[1,2] and research into the organism.[3-5] Although enzyme-linked immunosorbent assay (ELISA), which does not require live organisms, is widely used in toxoplasma diagnosis, the Sabin Feldman dye test remains the definitive serological test and is the gold standard.[2] The dye test measures the amount of total *T. gondii*-specific antibody and has the advantage of being both highly sensitive and specific.[2]

Historically, the requirement for live toxoplasma was met by the culture of *T. gondii* in rats or mice,[1,6] but recently we perfected a robust culture system in HeLa cells for the continuous production of *T. gondii* RH strain tachyzoites.[7] As with any cell culture method, however, it is vulnerable to failure for a variety of reasons, including poor cell growth or bacterial contamination.[8] In these circumstances it is crucial that it is supported by a reliable, rapid backup system.

The long-term storage of cells or microorganisms in liquid nitrogen is well established[9-11] and can be used when continuous culture fails. It can also be used to provide fresh material to replace cultures that have undergone excessive multiple passage.[12] However, unreliable retrieval of tachyzoites from these stores jeopardises a routine diagnostic service and compromises experimental use.

We are aware that several laboratories are considering use of toxoplasma cell culture for diagnosis, in particular with the dye test, or experimentation, and this study details methodology to ensure liquid nitrogen retrieval.

Materials and methods

Continuous toxoplasma culture

Continuous culture was performed in HeLa cells, as previously described.[7] Cell culture flasks (Corning, High Wycombe, UK) were seeded with HeLa cells (9×10^6 cells for 75 cm^2 flasks; 3×10^6 cells for 25 cm^2 flasks) in growth medium (Minimum Essential Medium Eagle with Earles Balanced Salt with 25 mmol/L Hepes solution [EMEM Hepes; Bio Whittaker, Wokingham, UK]) supplemented with L-glutamine (2 mmol/L; Bio Whittaker), gentamicin (40,000 units; Roussel Laboratories, Uxbridge, England), fungizone (1 mg/mL; Squibb and Sons, Hounslow, England) and 10% fetal calf serum (FCS; Bio Whittaker), and incubated at 37°C with 5% CO_2 for 6 h to form a monolayer.

The cells were changed to maintenance medium (as growth medium with 2% FCS) and incubated at 37°C with 5% CO_2 for 16 h before infecting with viable RH strain *T. gondii* tachyzoites (9×10^6 for 75 cm^2 flasks and 3×10^6 for 25 cm^2 flasks). After 24 h the medium was replaced with serum-free medium.

Toxoplasma tachyzoites form part of the asexual cycle and represent the replicating form of the parasite found in human disease.[13] Crescentric in shape and measuring 2–4 x 4–7 μm, they follow a predictable pattern of cell invasion, rapid multiplication within, and release from, the cell.[13]

Cultures were examined daily by phase contrast microscopy using an inverted microscope to determine the presence of free tachyzoites. Flasks were incubated at 37°C with 5% CO_2 until maximally infected (>5 plaques/field

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at x400 magnification; 48–54 h post-infection) and then transferred to 25°C. Flasks were harvested up to 168 h later and harvests considered successful if they met the criteria of ≥ 1 x 10^6 tachyzoites/mL, ≥ 90% viable.

Routine freezing in liquid nitrogen

T. gondii tachyzoites were frozen in liquid nitrogen using a modification of the method of Kozojed *et al*.[11] Harvests containing 3–6 x 10^6 tachyzoites/mL, ≥ 90% viable were selected and frozen as soon as possible after harvest. Samples were centrifuged at 1000 rpm for 10 min in a Jouan GR422 centrifuge at 4°C. A chilled freezing solution (growth medium, 10% DMSO [Sigma Aldrich Co Ltd, Irvine, UK] and 10% FCS) was freshly prepared and kept on ice. Tachyzoite pellets were resuspended in 1.8 mL freezing solution and transferred to cryotubes (Nunc, Rostilde, Denmark) on ice. Suspensions were frozen to –30°C at a rate of –1°C/min, then stored frozen in liquid nitrogen. The total number of tachyzoites in the cryotubes ranged from 1.5–6 x 10^7.

Recovery of tachyzoites from liquid nitrogen

Cryotubes were removed from liquid nitrogen and the suspensions thawed rapidly and added immediately to confluent HeLa monolayers in 25 and 75 cm^2 flasks (one cryotube/flask) in maintenance medium (10 mL and 20 mL, respectively) and incubated at 37°C with 5% CO_2. The medium in the flasks was replaced with fresh maintenance medium after 6 h, and then with serum-free medium after 24 h.

Cultures were examined microscopically on a daily basis for tachyzoite growth and biofilm stability, and passed blind every three or four days until recovery. For blind passage, the biofilm was scraped from the flask surface using a plastic pipette and half of the suspension (5 mL or 10 mL) was used to infect a fresh HeLa monolayer. Usually, at least two blind passes were necessary, but as soon as harvests of ≥ 1 x 10^6 extracellular tachyzoites/mL, ≥ 90% viable were produced they were passed as for continuous culture and the quality of tachyzoites was assessed by their performance in the dye test.

Assessment

The dye test was performed using cell culture-derived tachyzoites as previously described[2] on pre-selected control sera. To perform satisfactorily, the dye test must yield the expected titres (±1 dilution) for each serum. The time in days taken to obtain tachyzoites that performed well in the dye test was taken as the recovery time.

Determination of optimum tachyzoite inoculum for recovery

Four harvests were each used to prepare one cryotube containing 5 x 10^7, 1 x 10^7, 1 x 10^6 and 1 x 10^5 viable tachyzoites in 1.8 mL freezing solution, as described previously. The cryotubes were frozen in liquid nitrogen for one week and each retrieved into a 25 cm^2 HeLa cell monolayer, as before. The first scraped harvest from each flask was then divided between two 25 cm^2 HeLa monolayers and each then re-passed until the harvest was of dye test quality.

Comparison of tachyzoite recovery in 25cm^2 and 75cm^2 flasks

Flasks (25 cm^2 and 75 cm^2) were infected with the optimum number of tachyzoites, determined from above. Three harvests that had been frozen in liquid nitrogen for nine days – seven months were used to infect duplicate HeLa monolayers with 1 x 10^7 tachyzoites for 25 cm^2 flasks and 3 x 10^7 tachyzoites for 75 cm^2 flasks, as described previously.

Cultures were maintained, scraped, harvested and passaged as for recovery from liquid nitrogen. Four flasks of each size were maintained and passaged as for continuous toxoplasma culture for a further two weeks to assess the sustainability of the recovered tachyzoites.

Removal of storage medium

Two cryotubes with 1 x 10^7 tachyzoites were removed from liquid nitrogen and rapidly thawed. The contents of one cryotube were centrifuged at 1000 rpm for 10 min and the tachyzoites resuspended in 10 mL maintenance medium and added to a 25 cm^2 HeLa monolayer. The contents of the other cryotube was added directly to a 25 cm^2 HeLa monolayer with 10 mL maintenance medium. The maintenance medium was replaced in this flask after 6 h incubation at 37°C in 5% CO_2 to remove the storage medium (including the DMSO). After a further 24 h incubation, both flasks were changed to serum-free medium and cultures maintained as for continuous culture. This experiment was carried out twice in duplicate flasks.

Results

The ability of different sizes of inocula retrieved from liquid nitrogen to produce a successful harvest in continuous cell culture is recorded in Table 1. The optimum total number of tachyzoites for retrieval into a 25 cm^2 flask of HeLa cells was 1 x 10^7. All the flasks inoculated with this number were successfully retrieved, whereas only 75% of the other inocula (5 x 10^7, 1 x 10^6 and 1 x 10^5) were successful. Furthermore, the mean time and mean number of passes to retrieval was shorter using an inoculum of 1 x 10^7 (Table 1).

Suspensions with a higher tachyzoites:HeLa cell ratio generally produced a quicker recovery. Two of the harvests that were frozen in liquid nitrogen had a tachyzoites:cell ratio >10:1, while the other two were <10:1. In all flasks, except those with inocula of 1 x 10^5, the time and number of passes to recovery were less for inocula with a >10:1 tachyzoites:cell ratio than a <10:1 ratio (Table 1).

Retrieval directly into 75 cm^2 flasks resulted in quicker recovery (mean: 8 days) than retrieval in 25 cm^2 flasks (mean: 9.6 days) (Table 2). Similarly, the number of passes taken for successful retrieval was less for 75 cm^2 flasks than for 25 cm^2 flasks (2.2 and 2.6 passes, respectively). After seven days following successful retrieval, the mean concentration of tachyzoites in 75 cm^2 flasks was 2.3 times that found in 25 cm^2 flasks (Table 2). After 14 days the concentration in 25 cm^2 flasks improved but the 75 cm^2 flasks were still 1.3 times higher. Viability of recovered harvests was better with 75 cm^2 flasks (mean: 98%) than with 25 cm^2 flasks (mean: 95%) (Table 2). All were successfully used in the dye test.

The use of centrifugation prior to infection to remove any potential soluble toxins present in the storage medium (including perhaps DMSO, as well as toxins released by dead/dying cells) resulted in complete failure of tachyzoite recovery. Tachyzoites were successfully recovered when potential toxins were removed by replacing the maintenance media 6 h after infection.

Table 1. Effect of the total number of tachyzoites in the inoculum to produce a successful harvest in cell culture following retrieval from liquid nitrogen.

Infecting inoculum				Tachyzoites:HeLa cell ratio in infecting inoculum					
				>10:1			<10:1		
Tachyzoites	Success	Days Mean (range)	Passes Mean(range)	Success	Days Mean (range)	Passes Mean (range)	Success	Days Mean (range)	Passes Mean (range)
5 x 10^7	6/8	10 (6-13)	3 (2-4)	2/4	6 (6)	2 (2)	4/4	11.5 (10-13)	3.5 (3-4)
1 x 10^7	8/8	9 (6-10)	2.75 (2-3)	4/4	7.5 (6-9)	2.5 (2-3)	4/4	10 (10)	3 (3)
1 x 10^6	6/8	11 (9-14)	3.33 (3-4)	4/4	9.5 (9-10)	3 (3)	2/4	14 (14)	4 (4)
1 x 10^5	6/8	11 (6-14)	3.33 (2-4)	2/4	13 (13)	4 (4)	4/4	10 (6-14)	3 (2-4)

Table 2. Effect of flask size on the success of retrieval from liquid nitrogen.

Flask size (cm^2)	No.	Time to recovery (days)	Passes to recovery	Count* (viability) of tachyzoites in harvests collected at specified times (days) after recovery	
				7	14
75	1	7	2	3.75 (98)	4.35 (98)
	2	11	3	4.60 (98)	4.30 (98)
	3	4	1	2.65 (98)	3.90 (98)
	4	4	1	2.70 (98)	4.90 (98)
	5	11	3	ND	ND
	6	11	3	ND	ND
25	1	11	3	1.35 (92)	3.40 (98)
	2	11	3	1.50 (90)	6.20 (95)
	3	7	2	1.65 (96)	1.70 (95)
	4	7	2	1.30 (98)	1.50 (95)
	5	11	3	ND	ND
	6	11	3	ND	ND

*count x10^6/mL

ND – not done

Discussion

An established method of freezing *T. gondii* tachyzoites suspended in growth medium and 10% DMSO[11] provides a robust method of storing tachyzoites from animal culture, even when tachyzoites have been maintained for several hours at 4°C. However, preliminary attempts to retrieve tissue culture-derived tachyzoites into continuous cell culture using this freezing method showed only 36% success.

Unlike *in vivo* cultured tachyzoites, cell culture parasites rapidly lose viability on storage at 4°C. Therefore, it is important to add chilled reagents to freshly harvested parasites immediately before controlled cooling to – 30°C. This improved freezing procedure only partly resolves the problem, however, increasing the success rate to 75%.

Here, the number of tachyzoites per frozen sample was found to be crucial for their successful recovery. Although larger numbers of tachyzoites may be expected to have a cryoprotective effect, failure of the inoculum to recover with 5 x 10^7 tachyzoites arose because the HeLa cell monolayer became infected too rapidly to be of use in the routine culture system.

Premature disintegration of the HeLa cell monolayer results in extracellular tachyzoites adhering to the plastic of the flask and dying. In contrast, smaller inocula with 1 x 10^6 and 1 x 10^5 tachyzoites failed to grow.

Early attempts at the retrieval of liquid nitrogen stocks of tachyzoites into cell culture relied on the use of 25 cm^2 flasks, with transfer to 75 cm^2 flasks once tachyzoite growth had become established. Success in the dye test after the recovered tachyzoites had been cultured for 14 days following recovery suggests that consistent production of good quality tachyzoites in 75 cm^2 flasks is sustainable following their initial recovery.

Although the number of flasks in the present comparison is too small for statistical analysis, the experiments demonstrated that direct recovery to 75 cm^2 flasks is at least as good as two-step recovery via 25 cm^2 flasks. The practical advantage of omitting a stage in the recovery procedure with 75 cm^2 flasks is that tachyzoites are available earlier.

Attempts to remove any potential soluble toxins by centrifugation prior to infection resulted in complete failure of tachyzoite recovery, presumably as a result of physical damage to the tachyzoites.[8] Early replacement of maintenance media (6 h post-infection) was the preferred

method as dye test-quality tachyzoites were recovered and potentially harmful insoluble cell debris were removed.

The introduction of blind passage has also been crucial in improving the *T .gondii* recovery rates to 100% in continuous cell culture, as it has allowed the procedure to become standardised and ensures that tachyzoites continually have access to healthy cells. Efficient retrieval of *T. gondii* tachyzoites from storage in liquid nitrogen for continuous cell culture requires more stringent protocols than those needed for culture in animals.

In this study, procedures have been optimised for freezing *T. gondii* tachyzoites, and for their retrieval from liquid nitrogen. This reliable and rapid retrieval of quality *T. gondii* tachyzoites from liquid nitrogen ensures a continuous cell culture system for the production of *T. gondii* tachyzoites for diagnostic or experimental use. □

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