Alexandria Journal of Medicine

ISSN: 2090-5068 (Print) 2090-5076 (Online) Journal homepage: https://www.tandfonline.com/loi/tajm20

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To cite this article: Mohamed M. El-Bahy, Marwa M. Khalifa & Eman M.H. Méabed (2018) *Toxoplasma gondii*: Prolonged in-vitro maintenance of virulent tachyzoites in fluid media at low temperatures, Alexandria Journal of Medicine, 54:4, 511-515, DOI: 10.1016/j.ajme.2018.10.006

To link to this article: https://doi.org/10.1016/j.ajme.2018.10.006

Alexandria Journal of Medicine 54 (2018) 511–515

HOSTED BY

Contents lists available at ScienceDirect

Alexandria Journal of Medicine

journal homepage: http://www.elsevier.com/locate/ajme

Original Article

Toxoplasma gondii: Prolonged in-vitro maintenance of virulent tachyzoites in fluid media at low temperatures

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ARTICLE INFO

Article history:
Received 30 September 2018
Accepted 20 October 2018
Available online 27 November 2018

Keywords:
Toxoplasma gondii
Tachyzoites
Fluid media
Temperature

ABSTRACT

Background: Prolonged maintenance of infective *Toxoplasma gondii* tachyzoites (*T.g.*T.) is an important subject for research purposes. This study aimed to evaluate four serum free fluid media for prolonged in vitro maintenance of *T.g.*T.
Methods: The four fluid media Phosphate buffered saline (PBS) *pH 7.2* and *Roswell Park Memorial Institute* (*RPMI-1640*) with or without 3% fetal bovine serum (FBS) were evaluated for maintenance of virulent *T.g.* T. The four media were tested after incubation at three different temperature degrees in the darkness.
Results: Prolonged maintenance period for infective *T.g.*T. was recorded especially in the absence of FBS supplement. RPMI without FBS was able to maintain infective *T.g.*T. for 16 days post incubation (dpi) at refrigerator temperature. This period decreased to 10 dpi and 6 dpi after incubation in the same media at 18–22 °C and 37 °C, respectively. Cultivation of *T.g.*T. in RPMI supplemented with 3% FBS and in PBS proved to maintain infective *T.g.*T. for 14 dpi at refrigerator temperature, and for 9 and 5 dpi when the two media were incubated at 18–22 °C and 37 °C, respectively. Shorter periods for keeping the *T.g.*T. infectivity were recorded using PBS supplemented with 3% FBS under all tested temperature conditions.
Conclusion: This method allows economic long-lasting maintenance of tachyzoites for 16th dpi in RBMI that can be reactivated by reinoculation in mice.

1. Introduction

Toxoplasmosis is a worldwide zoonotic disease caused by an obligatory intracellular parasite; *Toxoplasma gondii*.[1] The parasite is capable of infecting virtually all warm-blooded animals, but felids are the only known definitive host in which the parasite can produce oocysts which pass in their feces. Bradyzoites and tachyzoites are the only asexual forms present in the intermediate host.[2]

Toxoplasma gondii strains have been divided into three groups based upon genotype and biochemical isoenzyme analysis of animal and human isolates.[3] RH strain of *T. gondii* genotype I is the most virulent strain in mice. It was isolated in the USA by Albert Sabin from a 6-year-old boy who died of toxoplasmic encephalitis in 1939. This strain has been maintained in mice or in cell culture in many laboratories worldwide and has become the reference *T. gondii* strain.[4] Genetically, RH is one of the type I strains which are extremely virulent in mice. Mice were demonstrated as the best animal model for this strain.[5] This strain didn't develop or infect rats.[6]

Preservation and storage of viable tachyzoites are seemed to be necessary in several fields such as specific antigen production for serological diagnostic, immunization, therapeutic, biochemical, genetic and molecular researches.[3] However, the obligate intracellular nature of this parasite affects the results of various experimental procedures as the host cell antigenic components couldn't be separated from the parasite one the matter affecting the purity of the produced tachyzoites.[7]

Prolonged maintenance of infective *T.g.*T. is an important subject for keeping live and active tachyzoites. Inoculation of *Toxoplasma* into mice, egg embryos and culturing in cell culture media are the common methods of keeping the permanent source of active tachyzoites in the interested laboratories. Each of these methods has complications and difficulties; the high cost, equipped laboratory and well-trained technicians, the need for frequent passage every 2–3 days, personal permanent attendance as well as the ethical aspect of work on laboratory animals.[8] Maintenance of *Toxoplasma* strain by cryopreservation is another method

Peer review under responsibility of Alexandria University Faculty of Medicine.
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https://doi.org/10.1016/j.ajme.2018.10.006
2090-5068/

that needs the availability of tanks of liquid nitrogen but carries many hazards for laboratory workers.[9]

To avoid the above mentioned drawbacks in maintaining active tachyzoites, the present study was conducted to characterize the appropriate conditions for prolonged in-vitro maintenance of *T.g.* T. harvested from peritoneal fluids of inoculated mice in cell free-fluid media under different temperature in dark.

2. Materials and methods

2.1. Et***hical approval

The study steps and procedures were approved by the Institutional Animal Care and Use ethical Committee (IACUC) of Faculty of Veterinary Medicine-Cairo University.

2.2. Preparation of tachyzoites

Tachyzoites of virulent *T. gondii* (RH) strain were propagated by intraperitoneal injection in parasite-free albino Swiss mice. Four days post inoculation; mice were killed by cervical dislocation. Tachyzoites from the peritoneal cavity were collected after injection of 1 ml of 0.1 M. PBS, pH 7.2. The intracellular tachyzoites were released by passing the collected peritoneal exudates through a 27 gauge needle three to four times. The cellular debris was removed by low speed centrifugation (100 g for 5 min). After collection of the supernatant, the parasites in the supernatant were sedimented by centrifugation at 600 g for 10 min, then washed in Hank's Balanced Salt Solution (HBSS) supplemented with penicillin G Sodium (100 U) and dihydro-streptomycin (100 μg) per ml (Sigma-Aldrich, USA). The number of tachyzoites/ml was determined using a hemocytometer and adjusted to 10^4 active parasites/ml. The mean percentage of viable tachyzoites after the final purification was estimated after three counts, and then transferred directly to the tested fluid media.[10]

2.3. Tested fluid media

The efficacy of four liquid media in the maintenance of viable and infective *T. gondii* tachyzoites was evaluated. The tested media were:

Medium 1: Phosphate buffered saline (PBS) pH 7.2.

Medium 2: PBS supplemented with 3% FBS, (GIBCO, BRL, USA).

Medium 3: RPMI-1640 Medium Auto-Mod™ for Autoclaving (Sigma-Aldrich, USA).

Medium 4: PPMI-1640 supplemented with 3% FBS.

All media were supplemented with 100 IU of penicillin G Sodium/ml and 100 μg of dihydro-streptomycin/ml. The media were filtered through a 0.22 μm millipore filter and stored at 4 °C until use.

2.4. Cultivation condition

One ml containing 10^4 active parasites was transferred into the fluid culture flask and the volume was adjusted with the tested media to 30 ml/flask. The flasks were completely covered with aluminum foil to provide the dark conditions. Each medium was presented in sex flasks, which were allocated into 3 groups (each of two flasks). Groups from each media were incubated in three tested temperature degrees as 37 °C, room temperature (18–22 °C), and refrigerator temperature (4–6 °C).

2.5. Testing the infectivity of the incubated tachyzoites

From the 3rd day post incubation (dpi), after gentle shaking of the flask, aseptically, a daily sample (500 μ) was taken from each flask using a sterile syringe. The viability of tachyzoites was estimated by direct examination of several drops of the fluid on a glass slide and examined under the microscope (×400) after application of a cover-slip. Enough amount of the fluid was mixed with 1.0% trypan blue and then microscopically examined.[8] The viable tachyzoites excluded the stain, while the dead absorbed the dye and appeared dark blue in color under the phase-contrast microscope. The percent of active motile tachyzoites was calculated in 100 examined tachyzoites (3 replicates). The mean viability of each flask was estimated. When the recorded viability reached around 60%, an amount of 200 ml of the media containing tachyzoites was inoculated intra-peritoneally in six 18–22 g albino Swiss mice per test and the infectivity of tachyzoites was evaluated as described by Diab and El-Bahy.[10] The duration of the experiment and observation depend on the vitality of tachyzoites in each media.

2.6. Statistical analysis

Study data were entered into Microsoft Office Excel 2007 software then analyzed by the computer statistical package SSPS version 17. The viability percentage of *T.g.*T. was presented as a mean and standard deviation. Student t-test was used to estimate the statistical difference of tachyzoites viability within a group at different dpi. The paired t-test was used to determine the statistical difference between the dependant results (obtained by the same fluid medium at two tested temperatures). Oneway ANOVA test was used to compare the viability rates obtained by different media on the same day. Differences expressed as significant at a cutoff $P \leq 0.05$.[11]

3. Results

The lifespan of cultivated tachyzoites was longer in the absence of FBS from the media under all tested temperatures. There was a significant increase in *T.g.*T. vitality rates in absence of 3% FBS ($P \leq 0.001$). RPMI and PBS media without FBS supplement were able to maintain active infective tachyzoites with variable percentages for 6 and 5 dpi, at 37 °C, for 10 and 9 dpi at 18–22 °C, and for 16 and 14 dpi at 4–6 °C, respectively. By adding 3% FBS to RPMI & PBS media, the maintenance periods were 5 and 4 dpi at 37 °C, 9 dpi at 18–22 °C, and 14 and 13 dpi at 4–6 °C, respectively (Tables 1–3).

Survival period and infectivity of the maintained tachyzoites were generally longer in stages maintained in RPMI without serum than any other media. This was true for the three tested temperature ($P \leq 0.001$ ANOVA test, Tables 1–3).

At 37 °C, PBS, PBS + FBS, RPMI and RPMI + FBS were able to keep 55%, 55.5%, 42.7% and 45.6% of the incubated tachyzoites highly motile and infective for 5, 4, 6 and 5 dpi, respectively (Table1).

By decreasing the temperature to 18–22 °C, the lifespan of tachyzoites was increased to 9, 9, 10 and 9 dpi with variable percentages of survival (49.3%, 42.8%, 42.3% and 51%) in the four above media, respectively (Table 2).

Among the four tested fluid media, the longest survival time of the incubated tachyzoites was recorded for the groups maintained at 4–6 °C. Infective tachyzoites viability percentages were 48.7% at 14 dpi, 48.6% at 13dpi, 38.13% at 16 dpi and 45% at 14 dpi in PBS, PBS + FBS, RPMI and RPMI + FBS, respectively (Table 3). For all tested media, there was a significant increase in the lifespan and infective ability by lowering the temperature of incubation. The difference was significant when comparing the results at 37–18 °C, 18–4 °C and 37–4 °C ($P \leq 0.001$, paired t-test, Table 4).

Variable percentages of tachyzoites survived, but failed to induce successful infection in the exposed mice for different peri-

Table 1
Vitality % (mean ± SD) and infectivity of tachyzoites maintained in fluid media at 37 °C in the darkness.

Days post incubation (dpi)	PBS		PBS + 3% FBS		RPMI		RPMI + 3% FBS		P value[2]
	% of Viability	% mortality of mice	% of Viability	% mortality of mice	% of Viability	% mortality of mice	% of Viability	% mortality of mice	
2 dpi	100 ± 0	NP	100 ± 0	NP	100 ± 0	NP	100 ± 0	NP	≤ 0.001**
3 dpi	89.5 ± 2.12	NP	83.4 ± 1.1	NP	100 ± 0	NP	93.5 ± 2.12	NP	
4 dpi	73 ± 1.4	NP	55.5 ± 0.5	100	77.8 ± 1.8	NP	66 ± 1.3	100	
5 dpi1	55 ± 0.7	100	32.3 ± 1.0	0	68.2 ± 1.7	100	45.6 ± 0.1	100	
6 dpi	35.5 ± 0.7	0	21.7 ± 0.4	0	42.7 ± 1.14	100	31.6 ± 2.0	0	
7 dpi	20 ± 1.4	0	17.4 ± 0.84	0	32.9 ± 1.5	0	23.6 ± 1.95	0	
8 dpi	14.75 ± 0. 35	0	8.8 ± 1.3	0	22 ± 3.6	0	18.1 ± 1.3	0	
9 dpi	8.35 ± 0.5	0	0 ± 0	0	14 ± 0.4	0	11.5 ± 0.7	0	
P value[1]	0.005*		0.017*		0.002*		0.005*		

NP: the test was not performed at this level of infection.
P value[1*]: comparison of viability rate at different dpi within the same group (student t test).
P value[2**]: comparison of viability rate at the same dpi between different groups (oneway ANOVA).

Table 2
Vitality % (mean ± SD) and infectivity of tachyzoites maintained in fluid media at room temperature (18–22 °C) in the darkness.

Dpi	PBS		PBS + 3% FBS		RPMI		RPMI + 3%FBS		P value[2]
	% of Viability	% mortality of mice	% of Viability	% mortality of mice	% of Viability	% mortality of mice	% of Viability	% mortality of mice	
4 dpi	100 ± 0	NP	100 ± 0	NP	100 ± 0	NP	100 ± 0	NP	≤ 0.001**
6 dpi	85.1 ± 1.0	NP	73 ± 1.6	NP	87.5 ± 0.9	NP	76.5 ± 0.7	NP	
8 dpi	67.4 ± 1.8	100	53.5 ± 1.8	100	72.8 ± 1.0	NP	64.4 ± 2.2	100	
9 dpi	49.3 ± 1.5	100	42.8 ± 1.0	100	60.9 ± 1.2	100	51 ± 1.3	100	
10 dpi	34.5 ± 0.9	0	31.9 ± 2.4	0	42.3 ± 1.9	100	35.9 ± 0.18	0	
11 dpi	25.6 ± 0.5	0	15.3 ± 0.4	0	35.8 ± 1.1	0	18.5 ± 0.7	0	
12 dpi	15.0 ± 0.6	0	10.5 ± 0.7	0	22.4 ± 0.9	0	12.6 ± 0.6	0	
P value[1]	0.004		0.008		0.001		0.005		

NP: the test was not performed at this level of infection.
P value[1*]: comparison of viability rate at different dpi within the same group (student *t*-test).
P value[2**]: comparison of viability rate at the same dpi between different groups (oneway ANOVA).

Table 3
Vitality % (mean ± SD) and infectivity of tachyzoites maintained in fluid media at refrigerator temperature (+ 4–6 °C) in the darkness.

dpi	PBS		PBS + 3% FBS		RPMI		RPMI + 3%FBS		P value[2]
	% of Viability	% mortality of mice	% of Viability	% mortality of mice	% of Viability	% mortality of mice	% of Viability	% mortality of mice	
5 dpi	100 ± 0	NP	100 ± 0	NP	100 ± 0	NP	100 ± 0	NP	≤ 0.001**
7 dpi	92 ± 0.7	NP	88.9 ± 0.7	NP	94.3 ± 1.4	NP	90.5 ± 2.8	NP	
9 dpi	89.6 ± 2.3	NP	82.0 ± 0.04	NP	90 ± 1.4	NP	85.6 ± 2.1	NP	
10 dpi	82.5 ± 1.6	NP	73.1 ± 3.4	NP	84.2 ± 0.27	NP	76 ± 2.1	NP	
11 dpi	72.2 ± 1.8	NP	60.5 ± 2.1	100	76.5 ± 0.7	NP	66 ± 2.8	100	
12 dpi	64.4 ± 0.8	100	53.0 ± 2.9	100	68.13 ± 1.2	100	57.13 ± 2	100	
13 dpi	54.7 ± 0.5	100	48.6 ± 0.4	100	59.8 ± 1.6	100	51.0 ± 1.4	100	
14 dpi	48.7 ± 0.7	100	42.2 ± 1.1	0	50.5 ± 0.7	100	45 ± 0.0	100	
15 dpi	42 ± 0.8	0	35.5 ± 3.23	0	45.3 ± 0.4	100	38 ± 2.1	0	
16 dpi	33.6 ± 0.64	0	28.2 ± 1.70	0	38.13 ± 0.18	100	28.1 ± 2.7	0	
17 dpi	22.2 ± 1.3	0	15 ± 1.4	0	26.4 ± 0.9	0	19 ± 7.0	0	
18 dpi	10.4 ± 0.5	0	0 ± 0	0	12.2 ± 1.14	0	8 ± 4.2	0	
P value[1]	≤ 0.001		≤ 0.001		≤ 0.001		≤ 0.001		

NP: the test was not performed at this level of infection.
P value[1*]: comparison of viability rate at different dpi within the same group (student t test).
P value[2**]: comparison of viability rate at the same dpi between different groups (oneway ANOVA).

ods, These periods were 8–9 dpi at 37 °C, (Table 1), 11-dpi at 18–22 °C (Table 2), while it was extended up to 17–18 dpi in tachyzoites maintained at 4–6 °C, in the different media (Table 3).

4. Discussion

Maintenance of viable *T.g.*T. in the laboratory is important for several purposes such as specific antigen preparation, immunization, therapeutic, biochemical, genetic and molecular researches.[7] Tachyzoites are usually maintained by proliferation in the peritoneal cavity of mice. This way considered to be expensive as the inoculated mice survived the infection for only 4–6 dpi.[10] For these reasons, identification of an economic, easily established method to prolong the survival period of infective tachyzoites in the laboratory is considered as an important objective. In time, while earlier attempts science 50 years ago failed to maintain *T. gondii*

Table 4
The degree of significant difference between the results obtained at two different temperatures circumstances for the same fluid medium.

Groups	(P value for significance)		
	37–18 °C	18–4 °C	37–4 °C
PBS	0.001	≤ 0.001	≤ 0.001
PBS + 3% FBS	0.001	≤ 0.001	≤ 0.001
RPMI	0.001	≤ 0.001	≤ 0.001
RPMI + 3%FBS	0.001	≤ 0.001	≤ 0.001

Paired sample test.

in vitro without cell culturing, the present study, proved the successful ability of four fluid media to maintain the survival of infected *T.g.*T. test at 3 different temperatures. The obtained results revealed that decreasing the temperature of incubation with the absence of FBS from the media prolonged the survival period and maintained the infectivity of the incubated tachyzoites.

In comparison with PBS, and in the absence of FBS supplement, RPMI proved to be more efficient for keeping infective *T.g.*T. RPMI and PBS media without FBS supplement were able to maintain active infective tachyzoites by variable percentages for 6 and 5 dpi, at 37 °C, increased to 10 and 9 dpi at room temperature and to 16 and 14 dpi at refrigerator temperature in the darkness for both media, respectively.

The efficacy of low temperature degrees in the prolongation of *T.g.*T. survival was previously mentioned,[8,10,12] as they reported that the best temperature for long-term maintenance of *T.g.*T. was 4 °C. In the authors' opinion and in agreement with Weiss and Kim,[3] prolonged survival of tachyzoites under low temperature was explained by the trial of the parasite to cope with the extracellular environment away from its natural intracellular living environment, since the extracellular existence of tachyzoites hinders their ability to proliferate and reproduce. Tachyzoites in fluid media at 4 °C retain the minimum metabolic rate so it can survive longer especially in the darkness. Decreasing the metabolic activity will decrease the rate of accumulation of decomposed proteins that affect the pH, and hence affect the vitality of the parasite.[8] As Earlier studies during 1951 concluded that the resistance of T. gondii out of the host is quite limited and that the parasite can be maintained viable only for few hours at 23–25 °C in the extracellular medium. Other authors observed that exudates or infected tissues containing *T. gondii* maintained in Tyrode solution could show the organisms viable if kept at 4 °C for 7–14 days.[13]

Supplementation of both media with 3% FBS did not improve the infectivity of *T.g.*T. than that previously recorded in the absence of FBS. While the same effect of lowering the incubation temperature on the parasite longevity and infectivity was obvious. By adding 3% FBS to RPMI & PBS media, the maintenance periods were 5 and 4 dpi at 37 °C, increased to 9 and 9 dpi at room temperature and to 14 and 13 dpi at refrigerator temperature, respectively. Prolonged survival of tachyzoites in the fluid media without FBS supplementation was previously reported at the level of cell media,[14] and at the level of fluid media[8] as they found that PBS and normal saline (4 °C) media maintained Toxoplasma tachyzoites for 11 days in vitro. Another study demonstrated that *T.g.*T. could be maintained in routine laboratory solution with high serum component (PBS + 20% FBS), namely axenic medium, up to 24 h only, without loss of parasite infectivity and viability in both the in vivo and the in vitro systems.[7]

It was important to demonstrate that FBS is a complex nutritional supplement that is routinely used in cell culture media. FBS is considered to be an important supplement for the health of the cell line.[11,15] The major functions of serum in culture media are to provide hormonal factors stimulating cell growth and proliferation, transport proteins carrying hormones, minerals, trace elements and lipids. Moreover, it contains an attachment, spreading and detoxifying factors needed to maintain pH or to inhibit proteases either directly or indirectly.[16] Moreover, FBS contains a high amount of albumin, acting as a microcarrier which able to influence tachyzoites survival and prolongs the lifespan and infectivity of the incubated tachyzoites on the level of cell lines.[7] On the contrary with this, the presence of FBS in the media has several drawbacks, such as it contains several complement proteins that have a principal role in innate immunity influencing the results of immunoassays when added to culture cell lines.[15] FBS contains a diverse repertoire of protein-coding and regulatory RNA species; the majority of them are retained even after extended ultracentrifugation.[17] FBS contains some inhibitory factors such as endotoxins as well as the specific or cross-reacted antibodies that form a problem on the level of purification of the produced *T.g.*T. antigens affecting the outcome of immunoassays.[10] Also, the decomposition of the nutrient protein components of FBS by tachyzoites can change the pH of the media and adversely affect the vitality of the maintained tachyzoites.[8] With the intensive efforts to decrease the global demands for FBS and thus to avoid the its previous drawbacks, the present work was done using fluid media with a very low concentration of FBS (3% only) as supplement to provide the essential growth requirements to tachyzoites with minimum interference with the constituent and requirements of the used media.

In the present study two media of balanced osmotic pressure, free of FBS showed success for maintenance of infective tachyzoites these were PBS and RPMI. In this study, The RPMI was superior on PBS as regard the maintenance of infective tachyzoites. Our results disagreed with the results of Kalani et al.[8], since they proved that PBS, pH 7.2 prolonged tachyzoite longevity for 5, 8, 12 dpi more than RPMI 4, 7, 6 dpi at 37, 18, 4 °C, respectively. In authors' opinion the superiority of RPMI may be related to the composition of this medium as it uses a bicarbonate buffering system and contains many essential amino acids, vitamins, besides the inorganic salts and other components like D-Glucose and Glutathione which covers the parasite metabolic activities, while the PBS is totally devoid of any nutrients, vitamins, proteins and fats.[18]

The infectivity was assessed in mice if the viability rate reached 60%, since above this rate it was expected that the parasites were still active and retain infectivity. It was noticed that despite the increased longevity of the parasites, the infectivity was kept for shorter periods. Infectivity was maximally noticed in stages maintained in RPMI without serum than any other media. Asgari et al. reported a decreased infectivity of *T. gondii* due to prolonged in vitro passages of the parasite. They reported that it may be correlated with the rapid growths in the tissue culture.[6]

For conclusion the present study recommended maintenance of *T.g.*T. harvested after mice inoculation in RPMI fluid media under refrigerator temperature in dark for 14–16 dpi, then the tachyzoites were collected from the media and reactivated by inoculation in mice for new mice-media period. This method is considered to be more economical than most related published methods as it saves a large number of mice to maintain virulent *T.g.*T. in the laboratory.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Conflict of interest

The authors have declared no conflict of interest.

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