International Journal of Medical Laboratory 2021;8(1):27-34.

Original Article

Prolonged Laboratory Maintenance of *Toxoplasma gondii* Tachyzoites in Co-cultivation with the Bovine *Theileria annulata*-Infected Lymphoblastoids

Vahid Nasiri * **Ph.D.**

Department of Parasitology, Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Karaj, Alborz, Iran

Article history
Received: 26 Jul 2020
Accepted: 25 Sep 2020
Available online: 4 Mar 2021

Keywords
Lymphoblastoid
Tachyzoites
Theileria annulata
Toxoplasma gondii

A B S T R A C T

Background and Aims: In most of the studies, *Toxoplasma gondii* is maintained in laboratory mice or studied *in vitro* using non-lymphoid cell lines or primary mouse macrophages. The target of our research was to design a new axenic culture of *Toxoplasma gondii* tachyzoites to providing a sufficient quantity of them.

Material and Methods: *Theileria annulata*-infected lymphoblastoids, which had been maintained up to 260 sub-cultures to attenuate the *Theileria annulata*, were evaluated for their suitability to the cultivation of *Toxoplasma gondii* tachyzoites. This cultivation process was carried out continuously for up to 10 passages, and after each 5 sub-culture, 0.1 ml of culture suspension (1×10^6 tachyzoites) was inoculated to each BALB/c mouse.

Results: It was observed that the tachyzoites have attacked the lympho-blastoids, multiplied inside them, and many fresh tachyzoites with typical shape and gliding movement were present in the culture suspension. In all processes of cultivation, the pathogenesis of parasites remained stable, and they were able to proliferate in mice and eventually lead to the death of the animals.

Conclusions: We describe here a new protocol for prolonged maintenance of tachyzoites of *Toxoplasma gondii*, which is more efficient (both in terms of yield and cost (it does not need fetal calf serum)) than other traditional methods for maintenance of the parasite.

*Corresponding Author: Department of Parasitology, Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Karaj, Alborz, Iran. **Email:** v.nasiri@rvsri.ac.ir

Introduction

Toxoplasma gondii (*T. gondii*) is an obligate intracellular apicomplexan parasitic protozoa that infect any nucleated cell types in many vertebrates worldwide [1, 2]. It has a widespread distribution, and almost one-third of the world's total population is believed to be infected with toxoplasmosis [3]. Prolonged maintenance of the parasite's infective form is the main subject for increasing live and active tachyzoites for using it in further research. Inoculation of the parasite into the mice peritoneal, embryonated egg, and cell culture media is the current method of keeping many tachyzoites in the laboratories [4]. These different methods have some complications and difficulties such as the high cost, the need to equipped laboratory and well-trained personnel, the requirement for frequent passage every 2-3 days, personal permanent attendance, and the ethical aspect of research on laboratory animals [5]. Preservation of the parasites by cryopreservation is another manner that requires the accessibility of tanks of liquid nitrogen that carries many dangers for laboratory workers [6]. Besides, because the infected mice maintained the infection for only 4–6 days, parasite maintenance through a passage in mice is considered a costly procedure; thus, cell culture is more economical for propagating tachyzoites [7]. These parasites are obligate intracellular organisms, that depending on isolate type, proliferate with a replication time of 6 to 9 hours during *in vitro* cultivation, and infected cells generally rupture when they reach 64 to 128 parasites/cell [2]. Diversity of cell types, like transformed cell lines (HeLa, CHO, LM, Vero, MDBK, 3T3, etc.) and culturing procedures have been used to keep tachyzoites *in vitro* [8, 9]. A significant limitation in performing the parasite proliferation on monolayer cell lines is that they are anchorage-dependent, and thus these cell types could not proliferate without attachment to the cell surface [10].

The purpose of this study was to develop a procedure for the culture of *T. gondii* tachyzoites and determine whether *T. gondii* tachyzoites grown in the *Theileria annulata (T. annulata)*-infected bovine lymphoblastoid cells.

Materials and Methods

Preparation of tachyzoites of *T.gondii*

Mice infection

Tachyzoites of *T. gondii* isolates RH were grown and increased in number by intraperitoneal injection at 5 weeks old, female BALB/c mice. The peritoneal exudates of infected mice were centrifuged for 10 min at 1000 g, and the resulted pellets were washed and resuspended in phosphate-buffered saline, pH 7.2. Tachyzoites were counted by using a Neubauer chamber slide (Haemocytometer) and their viability were assessed by trypan blue dye exclusion method. The fresh and viable tachyzoites (1×10^6) were injected into the mice intraperitoneal, and after 3 to 4 days of infection, with appearing the symptoms of the disease, the peritoneal exudate of the infected mice was collected in phosphate-buffered

saline (PBS), pH 7.2, and centrifuged at 1000g for 10 min.

T. gondii infection in monolayer Vero cell culture

Monolayer Vero cells, that were received from the cell bank of Razi Vaccine and Serum Research Institute, were used in this study. After washing the Vero cells with PBS, they were incubated with 0.25% trypsin for 5 min at room temperature and to release the cells from the bottom of the flasks, tapped sharply. Subsequently, a fresh medium, Roswell Park Memorial Institute (RPMI)-1640 (Sigma-Aldrich, USA) with 10% fetal bovine serum, was added to the trypsinized cells at 37˚C. An appropriate amount of the cells were sub-cultured every 3–4 days into flasks with fresh medium, and the cultured cells were incubated at 37˚C to make the confluent monolayer cell lines. The confluent monolayer Vero cells were infected with *T.gondii* tachyzoites that were obtained from the peritoneum cavity exudate of the infected mice and the infected cells were cultivated at 37˚C for 24 h. The tachyzoites from the infected cell cultures were washed twice with PBS, counted and resuspended in PBS, and were used to infect *T. annulata*-infected lymphoblastoids.

Infection of *T. annulata*-infected lympho-blastoids with *T.gondii*

Bovine lymphoblastoids that were infected with attenuated merozoites of *T. annulata* were cultured in RPMI-1640 medium with 10% bovine serum at 37˚C. At the time of the new subculture of bovine lymphoblastoids ($1\times l0^6$ cells/ml), the *T. gondii* tachyzoites (1×10^7 cells/ml) were obtained from the infected Vero cells, were added to the bovine lymphoblastoids suspension, and incubated at 37˚C .The interaction of tachyzoites and lymphoblastoids was studied daily using an invert microscope and the Giemsa staining method. Shape, density, and intra or extracellular positions of the tachyzoites were evaluated. At the time that was needed to reach the peak of the number of the replicated lymphoblastoids, the subculture was done by mixing the intact replicated *T. annulata*-infected lymphoblastoid, *T. annulata*-infected lympho-blastoids containing tachyzoites and the fresh RPMI-1640 medium with 10% bovine serum, each of them, one-third of the final volume. This process was carried out continuously for up to 10 passages. Each experimental procedure was repeated three times.

Determination of *in vivo* infectivity of cultivated parasites

During 10 passages and after every 5 passages, to determine the *in vivo* infectivity and pathogenicity of the tachyzoites cultivated with *T.annulata*-infected lymphoblastoids, the cell suspensions were inoculated to the peritoneum of female BALB/c mice (6-7 weeks). Three groups of BALB/c mice were evaluated as follows: 5 mice were infected with $1 \times l0^6$ tachyzoites of the RH isolate of *T.gondii* maintained in *T. annulata*-infected lymphoblastoids, 5 mice were infected with $1 \times l0^6$ parasites cultivated in Vero cells as the positive control, and 5 mice inoculated with PBS serving as the negative control. This research was performed in accordance with the recommendations in the Guide for the Care, and Use of Laboratory Animals of the Razi Vaccine and Serum Research Institute, and all animals experiments

were approved by the Institutional Animal Care and Research Advisory Committee of the Razi Vaccine and Serum Research Institute based on the Specific National Ethical Guidelines for Biomedical Research issued by the Research and Technology Deputy of Ministry of Health and Medicinal Education of Iran.

Statistical analysis

A statistical analysis by using SPSS-18 was used to perform the various comparability of the results. The student's t-test evaluated the differences between the averages of the quantitative variables, and the p value of less than 0.05 was accepted as statistically significant.

Results

Qualitative evaluation of tachyzoites growth into the lymphoblastoids

One day after interaction and exposure of the cell, it was shown that the tachyzoites invaded into the lymphoblastoids and multiplied within them and, in many cases, formed clusters and caused lymphoblastoids to burst. Several parasitophorous vacuoles contained many groups of the tachyzoites that were arranged in rosettes shape due to the synchronous division were observed. In the invert microscope observed that the more than three-quarters of the lymphoblastoid were attacked by tachyzoites of *T. gondii*, and in the outer space of the lymphoblastoid, there were a large number of tachyzoites under multiplying and proliferation with typical rotational motions of the tachyzoites of *T. gondii*. Overgrowth was observed in all culture media on day 4 post-infection with many tachyzoites were floating in the culture supernatant. Parasite shapes remained in natural forms (id est. crescentic or oval, with one end pointed and the other end rounded) during the cultivation period. Figure 1 shows the multiplying tachyzoites inside and outside the *T. annulata*-infected lymphoblastoids in Giemsa staining after 5 days post-infection. This growth pattern was observed until the end of the 10^{th} passage.

Quantitative evaluation

As a quantitative point of observation, 10% of *T. annulata*-infected lymphoblastoids were infected with *T. gondii* tachyzoites on the first day after infection. The four days after infection, almost all *T. annulata*-infected lymphoblastoids were infected. Under the light microscope, large numbers of free extracellular tachyzoites were observed (Figure 2). The number of tachyzoites was doubled each day up to day 5 and then declined between days 6 and 7 (Figure 3). The maximum numbers of harvested tachyzoites were 2.8×10^7 cells/ml after 5 days post-infection. In all days post-infection, when all parameters were comparable statistically, there were significant differences between tachyzoites numbers ($p < 0.05$).

***In vivo* assays**

The infectivity of tachyzoites maintained in *T.annulata*-infected lymphoblastoids was evaluated during 10 passages. The study showed that in all processes of cultivation, the pathogenesis of parasites remained stable, and they were able to proliferate in mice and eventually lead to the death of the animals.

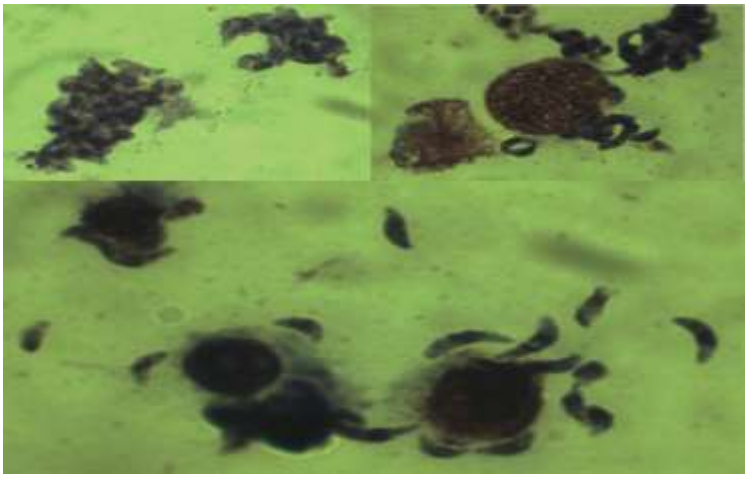

Fig 1. Multiplying tachyzoites of *T.gondii* inside and outside the *T. annulata*-infected lymphoblastoids in Giemsa staining.

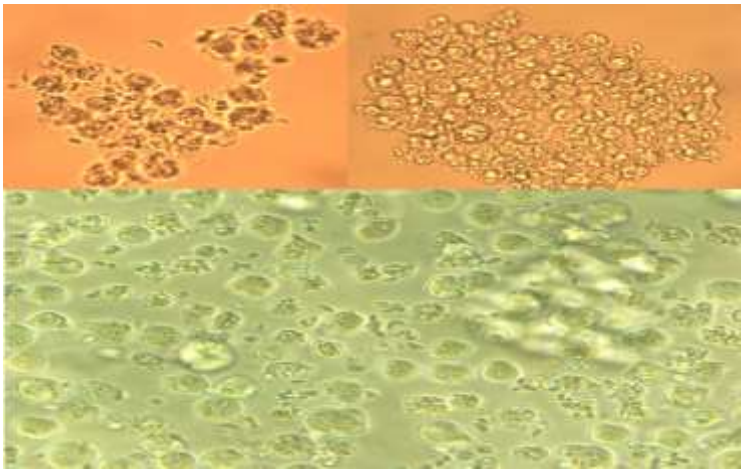

Fig 2. The tachyzoites of *T.gondii* in the inside and outside of the *T.annulata*-infected lymphoblastoids under the invert microscope, on day 5 of post infection. Numerous tachyzoites were observed throughout the culture.

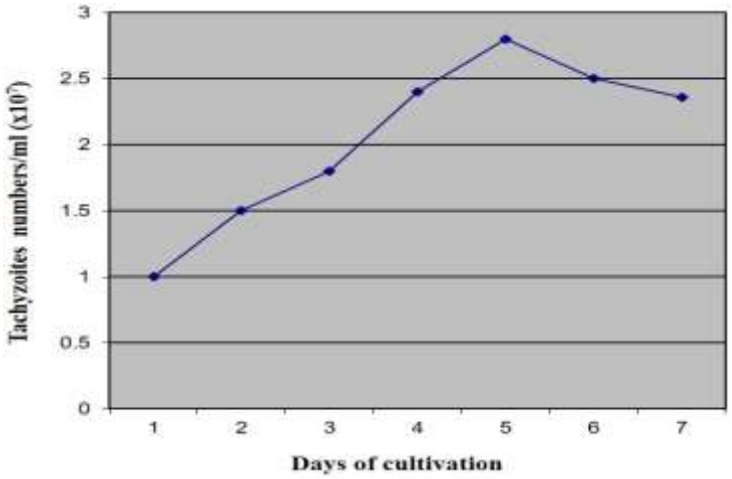

Fig 3. *In vitro* proliferation chart of *T.gondii* tachyzoites in *T.annulata*-infected lymphoblastoids cells.

In the infected mice, the typical characteristics of the *T. gondii* infection, including hispid hair, low physical activity, and apathy, were presented. 3-4 days post-infection of inoculated mice, free tachyzoites were presented in the peritoneal exudate of infected animals. The high parasitemia and mortality rates observed in mice infected with tachyzoites cultivated with the *T.annulata*-infected lymphoblastoids, which were similar to the control group that were infected with parasites cultivated in Vero cells in the same periods.

Discussion

Toxoplasma as an opportunistic parasite, could not continue its life without a host cell, thus, we are obligated to use different host cells to support its growth and maintenance. As a parasitic disease, toxoplasmosis is one of the significant causes of morbidity and mortality, especially in immune-compromised patients and the unborn fetus, and causes very significant economic losses to the livestock industry worldwide [11-13].

The different techniques of cells culture are approaches to make attempt to prepare complicated habitat situations of living organisms to develop our knowledge about their behavior and find appropriate methods, like an effective vaccine, to prevent adverse side effects of them [14]. The significant factors that affect the *in vitro* cultivation of *T. gondii* tachyzoites are the suitability and sufficiency of the nutritional requirements in the culture medium, the growth and behavioral characteristic of the host cell, and the multiplication rate of the parasite isolate and host cell. Optimizing a mass production technique means balancing factors such as the multiplication rate of the host cells and parasites, as well as, their generation periods under given culture conditions [15].

In some of the laboratories, the tachyzoites of *T. gondii* maintained by serial passages in the peritoneal cavity of mice. However, this method is ethically undesirable [16-19], thus, because cell cultures from different origins provide regular harvests of fresh viable tachyzoites, as an alternative method, many different mammalian cells such as HFF [20, 21], Hep-2 [6, 15], Vero [6, 8, 22, 23]; and HeLa [15, 16, 24, 25] that are sensitive to the *T. gondii* attack, are used for cultivation of this parasite [9, 15, 26]. In some researches have been indicated that a suspension culture of Hela cell has been a suitable host for *in vitro* cultivation of *T. gondii* tachyzoites [27], and other studies have been shown that a non-adherent cell line derived from TG 180 murine sarcoma cells could infect by tachyzoites of *T. gondii* and increased intracellularly [28]. Suspension culture could be used for many research applications and does not need enzymatic or mechanical separation, and is easier to passage and used for mass production and harvesting [29, 30].

Many years ago, as a congress oral presentation, we introduced a novel method for long-term maintenance of *T. gondii* tachyzoites in *Theileria anuulata*- infected and transplanted bovine lymphoblastoids [31].

After that presentation, in another study, researchers found that *Neospora caninum* tachyzoites could invade and proliferated well in the *T. annulata*-infected lymphoblastoids, suggest that a suspension culture of non-adherent cell lines of *T. annulata*-infected lymphoblastoids are appropriate to host cells for *in vitro* cultivation of *N. caninum* tachyzoites and are offered, as a viable alternative to the cultivation of the parasite *in vitro*. They indicated that, their work is the first report of suspension culture of *N. caninum* tachyzoites with *T. annulata*-infected cell line [10].

An important point in our research was that bovine serum was very suitable for the nutritional requirement of *T. gondii* and was a comparatively simply available and inexpensive serum that could be replaced in the media that require fetal calf serum enhancement and indicated to a potent new medium that did not need expensive fetal calf serum and could be used in long-term *in vitro* cultivation of *T. gondii* [31]. It should be mentioned that another advantage of our cultivation method is that *Theileria anuulata*- infected and transplanted bovine lymphoblastoids, as the non-adhesive cell lines, could be easily cultivated in fermenters that enable us to cultivate parasite in large scales that could be leading to mass production of probable vaccines. We must indicate that using this novel cultivation system, also accompanied by some little difficulties such as adaptation obstacles of the parasite to new serum and cell line. However, this issue takes place in any medium change, and the important point is the final adaptation of parasites to the novel culture system.

Conclusion

This research has introduced an alternative novel cultivation system that could be used for the usual and mass cultivation of *T. gondii* that enables us to reach suitable amounts of parasites for our different purpose. Further biochemical, physiological, and molecular studies need to show the details of the mechanism of this type of host-parasite interaction that could lead us to find new ways of controlled cultivation of the parasite in this type of cell.

Conflict of Interest

We have no conflict of interest to declare.

Acknowledgment

The author would like to thank all those who helped in carrying out this research.

References

[1]. Kim K, Weiss LM. Toxoplasma gondii: the model apicomplexan. Int J Parasitol. 2004; 34(3): 423-32.
[2]. Khan A, Grigg ME. Toxoplasma gondii: laboratory maintenance and growth. Curr Protocols Microbiol. 2017; 44(1): 20C-1.
[3]. Tenter AM, Heckeroth AR, Weiss LM. Toxoplasma gondii: from animals to humans. Int J Parasitol. 2000; 30(12-13): 1217-258.
[4]. El-Bahy MM, Khalifa MM, Méabed EMH. Toxoplasma gondii: prolonged in-vitro maintenance of virulent tachyzoites in fluid media at low temperatures. Alexandria J Med. 2018; 54(4): 511-15.
[5]. Kalani H, Daryani A, Sharif M, Ahmadpour E, Alizadeh A, Nasrolahei M, et al. Comparison of

eight cell-free media for maintenance of Toxoplasma gondii tachyzoites. Iran J Parasitol. 2016; 11(1): 104.
[6]. Diab MR, El-Bahy MM. Toxoplasma gondii: virulence of tachyzoites in serum free media at different temperatures. Exp Parasitol. Elsevier; 2008;118(1):75-9.
[7]. Zhao B, Wang Y, Wu B, Liu S, Wu E, Fan H, et al. Placenta-derived gp96 as a multivalent prophylactic cancer vaccine. Scientific reports. 2013; 3(1): 1-7.
[8]. Saadatnia G, Haj Ghani H, Khoo BY, Maimunah A, Rahmah N. Optimization of Toxoplasma gondii cultivation in VERO cell line. Trop Biomed. 2010; 27(1): 125-30.
[9]. Evans R, Chatterton JMW, Ashburn D, Joss AWL, Ho-Yen DO. Cell-culture system for continuous production of Toxoplasma gondii tachyzoites. Eur J Clin Microbiol Infect Dis. 1999; 18(12): 879-84.
[10]. Kargar M, Mojaver S, Namavari M, Sayari M, Rahimian A. Suspension culture of Neospora caninum by theileria annulata-infected cell line. Trop Biomed. 2013; 30(2): 349-54.
[11]. Wang ZD, Wang SC, Liu HH, Ma HY, Li ZY, Wei F, et al. Prevalence and burden of Toxoplasma gondii infection in HIV-infected people: a systematic review and meta-analysis. Lancet HIV. 2017; 4(4): 177-88.
[12]. Sensini A. Toxoplasma gondii infection in pregnancy: opportunities and pitfalls of serological diagnosis. Clin Microbiol Infect. 2006;12(6): 504-12.
[13]. Dubey JP. Toxoplasmosis of animals and humans. CRC press; 2016.
[14]. Nasiri V. An overview of the recent findings in the cultivation of Leishmania. Rev Med Microbiol. 2017; 28(1): 34-42.
[15]. Hughes HPA, Fleck DG. In vitro culture of Toxoplasma gondii in primary and established cell lines. Int J Parasitol. 1986; 16(4): 317-22.
[16]. Ashburn D, Evans R, Chatterton JMW, Joss AWL, Ho Yen DO. Toxoplasma dye test using cell culture derived tachyzoites. J Clin Pathol. BMJ Publishing Group 2000; 53(8): 630-33.
[17]. Benoit-Vical F, Santillana-Hayat M, Kone-Bamba D, Mallie M, Derouin F. Anti-Toxoplasma activity of vegetal extracts used in West African traditional medicine. Parasite 2000; 7(1): 3-7.
[18]. Petersen E, Pollak A, Reiter-Owona I. Recent trends in research on congenital toxoplasmosis. Int J Parasitol. 2001; 31(2): 115-44.
[19]. Villavedra M, Rampoldi C, Carol H, Baz A, Battistoni JJ, Nieto A. Identification of circulating antigens, including an immunoglobulin binding protein, from Toxoplasma gondii tissue cyst and tachyzoites in murine toxoplasmosis. Int J Parasitol. 2001; 31(1): 21-8.
[20]. Molestina RE, El Guendy N, Sinai AP. Infection with Toxoplasma gondii results in dysregulation of the host cell cycle. Cell Microbiol. 2008; 10(5): 1153-165.
[21]. Gail M, Gross U, Bohne W. Transferrin receptor induction in Toxoplasma gondii-infected HFF is associated with increased iron-responsive protein 1 activity and is mediated by secreted factors. Parasitol Res. 2004; 94(3): 233-39.
[22]. Costa-Silva TA, Meira CS, Ferreira IMR, Hiramoto RM, Pereira-Chioccola VL. Evaluation of immunization with tachyzoite excreted–secreted proteins in a novel susceptible mouse model (A/Sn) for Toxoplasma gondii. Exp Parasitol. 2008; 120(3): 227-34.
[23]. Meira CS, Costa-Silva TA, Vidal JE, Ferreira IMR, Hiramoto RM, Pereira-Chioccola VL. Use of the serum reactivity against Toxoplasma gondii excreted-secreted antigens in cerebral toxoplasmosis diagnosis in human immunodeficiency virus-infected patients. J Med Microbiol. 2008; 57(7): 845-50.
[24]. Chatterton JM., Evans R, Ashburn D, Joss AW., Ho-Yen D. Toxoplasma gondii in vitro culture for experimentation. J Microbiol Methods 2002; 51(3): 331-35.
[25]. Değirmenci A, Döşkaya M, Caner A, Çiçek C, Korkmaz M, Gürüz Y, et al. Toxoplasma gondii RH Ankara: Production of evolving tachyzoites using a novel cell culture method. Exp Parasitol. 2011; 128(1): 1-8.
[26]. Guimaraes FN, Meyer H. Cultivo de" Toxoplasma" Nicolle & Manceaux, 1909, em cultura de tecidos. Rev Bras Biol. 1942; 2(1): 123-29.
[27]. Valkoun A, Cinátl J. Propagation of Toxoplasma gondii in suspension cultures of HeLa cells. Folia Parasitol (Praha). 1979; 26(2): 165-67.
[28]. Couatarmanach A, Andre P, Le Minous D, Martin L, Robert R, Deunff J. In vitro culture and cloning of Toxoplasma gondii in a newly established cell line derived from TG180. Int J Parasitol. 1991; 21(1): 129-32.
[29]. Assoian RK. Anchorage-dependent cell cycle progression. J Cell Biol. 1997; 136(1): 1-4.
[30]. Griffiths B. Scale-up of suspension and anchorage-dependent animal cells. Mol Biotechnol. 2001; 17(3): 225-38.
[31]. Nasiri Vahid, Paykari Habibollah, Morrovati Hassan KG. A novel method for long-term maintenance of Toxoplasma gondii tachyzoites in Theileria anuulata- infected and transplanted bovine lymphoblastoids. The 4th Iranian Congress of Laboratory & Clinic. Tehran; 2011. p. 145.