Original Article

In Vitro and In Vivo Potential of RH Strain of *Toxoplasma gondii* (Type I) in Tissue Cyst Forming

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Abstract

**Background:** Based on recent studies, there are controversial reports on the capacity of tissue cyst forming of *Toxoplasma gondii* RH strain. In this study, the capacity was evaluated by *in vivo* and *in vitro* experiments.

**Methods:** RH strain was subcutaneously inoculated to ten Wistar rats. After one month, their blood, brain, tongue and diaphragm were collected and evaluated by MAT, PCR, pathological and bioassay methods. The parasite was cultivated in the cell monolayer. To change to bradyzoite, the media pH was altered to 6.8. Biological aspect of the bradyzoites was evaluated by incubation in acidic pepsin and it’s inoculation in ten BALB/c mice.

**Results:** All rats showed antibodies to *Toxoplasma* at titers ≥1:320 but no DNA and tissue cyst were detected in the tissues. Following intraperitoneal inoculation of rats’ brain homogenate into BALB/c mice, no infection was established in none of the animals. During presence of cell culture, in acid media for a 3-5 days period, cyst-like structures were noticed when they were stained with PAS. The visible bradyzoites in the cysts that were incubated in acid pepsin medium were not able to kill any mice.

**Conclusion:** This study confirmed that Iranian RH strain has lost the potential of tissue cyst forming in rats and bradyzoites cultivated in cell culture lost their resistance to acidic condition, so this strain can be a candidate for future vaccine researches.
Introduction

Toxoplasma gondii as an obligate intra-cellular protozoan can infect man and a wide range of warm-blooded animals (1). Infection in human hapens due to ingestion of oocysts from the feces of contaminated cats (2, 3) and by ingestion of raw or under-cooked tissue cyst containing products (4,5). Tachyzoites of T. gondii were noticed in dairy products of cows, sheep, goats, cats and mice (6-8).

Mice were demonstrated as animal model of toxoplasmosis due to sensitivity to the disease (9). Rats were reported as a resistant host model and were shown to be a suitable animal model for human toxoplasmosis (10). In the last two decades, cell culture systems have also been introduced as an alternative for animal models reducing the costs and ethical limitations (11) that were intended for diagnostic assays, vaccine strategies, drug sensitivity tests and other proposes such as in biochemistry, genetics and immunology (12,13).

Isolation of RH strain of T. gondii genotype I in a 6-year-old boy was first reported in 1939 (14) and has been passaged in mice and cell culture in many laboratories worldwide (15). The genotype I of T. gondii was shown to be responsible for lethal infections in outbred mice while types II and III were significantly less virulent (16).

Due to the prolonged passage of this strain, its pathogenicity was stabilized in mice (17), while this strain lost its potential to produce oocysts in cats (18). There are still controversies on the potential of cyst formation of this strain (19-21). It was shown that the RH strain lost its potential to tissue cyst formation in rats due to long passage time of tachyzoites (22). However, a report revealed that in mice, atovaquone together with pyrrolidinedithiocarbamate could change RH tachyzoites of T. gondii into tissue cysts (23). In Iran, some researchers presented evidences for tissue cyst formation of this strain in rat (24, 25).

This study determines the in vitro and in vivo potential of RH strain of T. gondii (Genotype I) in tissue cyst forming in rats.

Materials and Methods

Animals

Inbred BALB/c mice (6-8 weeks old weighted 22-25 grams) were provided from Pasteur Institute, Tehran, Iran. Two months old male Wistar albino rats (weighing 150-180 g) were obtained from the Laboratory Animal Center of Shiraz University of Medical Sciences, Shiraz, Iran. Animals were housed in cages and maintained under controlled conditions (21±2°C, 65-70% humidity and standard food and water ad libitum) during the experiments. The experiments were undertaken based on guidelines of laboratory animals in research and teaching book (26).

Parasite

The virulent RH strain of T. gondii was obtained from Tehran University of Medical Sciences, Tehran, Iran. Tachyzoites of this strain were collected by serial intraperitoneal passages in BALB/c mice. Parasites (1×10⁵) were inoculated in the mice, and after 72 hours, tachyzoites were provided by repeated flushing of the peritoneal cavity by Phosphate Buffered Saline (PBS). Tachyzoites were then harvested and centrifuged at 200 g for 5 min at room temperature to remove peritoneal cells and cellular debris. The supernatants were collected and centrifuged at 800 g for 10 min (21). The pellets, enriched with parasite tachyzoites, were recovered with PBS and used in the experiments.

Inoculation of parasite into rats

The tachyzoites (1×10⁵) of the virulent RH strain were subcutaneously inoculated to 10 albino Wistar rats. After one month, blood, brain, tongue and diaphragm tissues were evaluated by Modified agglutination test (MAT),
Polymerase chain reaction (PCR), pathological and bioassay methods.

**Modified agglutination test (MAT)**

Blood samples of the animals were collected and the sera were separated. The MAT was performed using formalin fixed whole tachyzoites and mercaptoethanol as previously described Dubey and Desmonts in 1987. A positive reaction at a 1:20 dilution of sera was indicative of previous exposure to the parasite (27).

**Histological examination**

To confirm the presence of tissue cyst of the parasite in brain, tongue and diaphragm of rats, the animals were euthanized by ketamine and xylazine. Sampling was undertaken via autopsy and fixed in 10% buffered formalin for histological evaluation stained by hematoxylin and eosin at 100x and 400x magnifications.

**Bioassay in mice**

The brain tissues from rats were first homogenized and then intraperitoneally inoculated into 5 mice. If the mice died, their liver impression smears were stained by Giemsa dye and investigated under light microscopy for parasite detection. One month after the inoculation, crush and impression smears were prepared again from liver, spleen and brain tissues of live mice and were parasitologically examined for presence of tachyzoites or tissue cysts.

**DNA extraction**

A total of 30 samples were taken from brain and diaphragmatic tissues of the rats. For extraction of DNA, 200 mg of these organs were homogenized and then diluted with double-distilled water (1:10). Proteinase K (10 µL) and lysis buffer (50 ml of Tris–HCl, pH=7.6; 1 mM of EDTA, pH=8.0; 1% Tween 20) were added to 500 µL of each sample and the samples were incubated for 24 h at 37°C. The lysate was extracted with phenol/chloroform/isoamylalcohol and then with chloroform/isoamylalcohol solutions. The DNA was precipitated with absolute ethanol and then was resuspended in 100 µL of double-distilled water and stored at 4°C until use.

**Nested PCR**

Nested primer sets (Bioneer, Korea) were used for amplifying fragments of the B1 gene as previously described (28). The external primers were 5′-GGA ACT GCA TCC GTT CAT GAG-3′ and 5′-TCT TTA AAG CGT TCG TGG TC-3′ producing an amplified product of 193 bp.

All the PCR reactions were performed in a programmable thermocycler (Eppendorf, Mastercycler gradient). The first 25 µL of PCR reaction mixture contained outer primers at a final concentration of 50 pmol each, 2.5 mmNTPs, 1 µg of template, and 1.5 U recombinant taq DNA polymerase (GENET BIO, Korea, A-type prime TaqTM DNA polymerase), in 1× PCR reaction buffer (50 mmol/L KCl and 10 mmol/L Tris–HCl, 1.5 mmol/L MgCl2, and 0.1% triton X-100; Sinagen Co., Iran). The first step of amplification was 5 min of denaturation at 94°C. This step was followed by 40 cycles, with one cycle consisting of 10 seconds at 94°C, 10 seconds at the annealing temperature (57°C) for each pair of primers, and 30 seconds at 72°C. The final cycle was followed by an extension step of 10 min at 72°C. Nested reactions contained 1 µL of the first-round product, 10 mMTris–HCl at pH 8.3 and 25°C, 50 pmol each, 2.5 mmNTPs, 1 µg of template, and 1.5 U recombinant taq DNA polymerase. Internal primers were 5′-TGCATAGGGTTGCAGTCAGTG-3′ and 5′-GGCGACCAATCTGCGAATACC-3′ producing an amplified product of 96 bp. Nested PCRs cycled 40 times using a denaturation step of 93°C for 10 seconds, followed by annealing at 62.5°C for 10 seconds and extension at 72°C for 15 seconds. Negative control samples from first-round amplification and an additional second-round negative control of sterile water were included in the nested reactions. The extracted DNA from RH strain of *T. gondii* was utilized as positive control. The amplification products were detected by gel elec-
trophoresis using 2% agarose gel in 1× Tris–borate–EDTA buffer. DNA bands were visualized in the presence of ultraviolet light, following the staining with 0.5% ethidium bromide.

**Cell culture**

HeLa Cells obtained from the Immunology Department, Shiraz University of Medical Sciences, were grown in 5 ml of culture medium using 25 cm² flasks (Corning Costar UK, UK). A monolayer of the cells were provided in RPMI 1640 with 10% heat-inactivated fetal calf serum (FCS; Gibco Company, USA) and 100 IU/ml penicillin–100 μg/ml streptomycin (Roche Company) and incubated in 5% CO₂ at 37°C and >80% humidity. Cells were routinely subcultured every 3 days by trypsination and washing with phosphate-buffered saline (pH=7.2).

**Toxoplasma gondii tachyzoites**

After 70% confluency of the cell line in a monolayer condition, the tachyzoites were added to the cell culture in a ratio 3 to 1. The flasks were then incubated at 37°C, >80% humidity and 5% CO₂ for 24 h.

**Toxoplasma gondii bradyzoite and tissue cyst formation in cell culture**

After 70% confluence of the cell monolayer of the line, pH of the media was reduced to 6.8. Then *T. gondii* RH strain tachyzoites, in a ratio 3 to 1 were added to the cell culture. After 8 hours, when active tachyzoites had entered into the cells, the media were replaced. Afterwards, the media were replaced and their pH media were adjusted every 48 hours.

**Periodic Acid Schiff (PAS)**

After removal of the infected cell culture media, the bottom of the flask was cut and dried. Then it was fixed in absolute methanol, allowed to dry, oxidized in 0.5% periodic acid solution for 5 minutes, rinsed in distilled water, placed in Schiff reagent for 15 minutes (changed into light pink color), washed in warm tap water for 5 minutes (changed into dark pink color), counterstained in Mayer's hematoxylin for 1 minute, washed in tap water for 5 minutes and finally dehydrated and covered with a slip using a synthetic mounting medium.

**Infectivity of tachyzoites and bradyzoites grown in cell culture**

The 1×10⁵ tachyzoites obtained from the cell culture were intraperitoneally inoculated into 5 mice. As control group, the same number of parasites obtained from intraperitoneal passing in BALB/c mice was intraperitoneally inoculated into another five mice. In the other experience, on the 5th day, the supernatant of the flask was removed and the precipitate was washed with phosphate-buffered saline (pH=7.3) and centrifuged at 1200 rpm for 10 min. Three milliliter of acid pepsin solution (pepsin, 2.6 g; NaCl, 5.0 g; HCl, 7.0 ml and distilled water to make 500 ml, pH ~ 1.10-1.20) as described before (29) was added to the sediment, incubated at 37°C for 60 min and then the homogenate was neutralized with 1.2% sodium bicarbonate. One milliliter of the neutralized homogenate was then intraperitoneally inoculated into each of the five mice.

**Results**

After one month of inoculation by RH strain of *T. gondii*, all rats showed antibodies titer ≥1:320. Histologically, no tissue cyst was observed in their brain, tongue and diaphragmatic tissues. Also, no DNA of the parasite was detected in these organs and inoculation of the brain tissue homogenate could not kill any of the BALB/c mice.

In necropsy of the mice, neither tachyzoite nor tissue cyst were visible in crush smears of their brain and liver tissues. During presence of cell culture, in acid media for a 3-5 days period, cyst-like structures were noticed when they were stained with PAS. Figure 1 shows the organisms in red color, demonstrating several amylopectin granules in the organism. After
incubation of the bradyzoites in acid pepsin solution and inoculation into BALB/c mice, all mice were still alive and the cyst and bradyzoites in the cell culture were all sensitive to acid-pepsin based on bioassay in mice.

Discussion

In this study, no tissue cyst and even DNA parasite were detected in brain, tongue and diaphragmatic tissues of rats. In addition, infection of mice was not successful after inoculation of a homogenate of brain tissue of the infected rats into the mice. Our findings were indicative of the fact that the RH strain of T. gondii was not capable of any tissue cyst formation in rats but could lead to high titers of antibodies against the parasite. It seems that the immune system responses results into removal of the parasite from the whole body of the host.

The authors in another study (Unpublished data) showed that when RH strain of the parasite tachyzoites were inoculated into rats treated with cyclophosphamide, all animals died within 5-7 days and tachyzoites could be recovered from the autopsy specimens. The virulence of the parasite in a host is dependent on several factors such as stage of the parasite, route and dose of administration and the genetic of the parasite (30). Dubey and Beattie revealed that the infection in intermediate hosts due to ingestion of the parasite oocysts were usually associated by more lesions in comparison to tachyzoites or tissue cysts (31).

Rat as an animal of choice for induction of chronic infections was shown to be infected even with only one live Toxoplasma tachyzoite (22). In several surveys on rats, the parasite was rarely noticed in histological sections of the infected animal with the RH strain (32, 33). In the study of Benedetto et al. (34), no tissue cysts were detected in brain of rats inoculated with the RH strain of the parasite. However, they showed that when the rats were treated with corticosteroids or were irradiated, clinical manifestations of toxoplasmosis were visible in the animals and small numbers of tissue cysts were found (34).

Moreover, it was clear that the passage of RH strain in rats resulted into attenuation of tissue cysts/bradyzoites (35). Tachyzoites of RH strain of the parasite could not lead to formation of tissue cysts in skeletal muscle (21). RH strain of the parasite lost its potential for formation of tissue cysts due to the prolonged passages of tachyzoites (26). For the first time, Sraei et al. (36) proposed that the Iranian strain has lost its ability of cystogenesis in rats.

Djurkovic-Djakovic et al. showed an association between the parasite and atovaquone and pyrroldinedithiocarbamate for changing RH strain tachyzoites of T. gondii into tissue cysts in mice (23). The cysts of the RH strain detected in the brain of mice were shown not to be able to infect mice via the oral transmission because of previous treatment with sulfadiazine. Using immunofluorescence assays revealed no change for tachyzoite into bradyzoites (19).

The difference in pathogenicity of the RH strain in rats may be correlated with the genetic background of the parasite that happened during the prolonged passages in mice (37). Weiss and Kim reported a decrease in cyst formation in avirulent isolates 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 (20). Also, the prolonged in vitro passages of other Apicomplexa such as Besnoitia jellisoni may result into the loss of the ability of tissue cyst formation in mice (18). In another study, it was demonstrated that S-48 as a live attenuated parasite strain was not able to produce any oocyst and tissue cyst which was reported to be a candidate for vaccines with commercialized veterinary purposes (38).

In our study, complementary tests were undertaken to evaluate the changes of tachyzoites into bradyzoites in cell cultures. To stimulate changing of tachyzoites into bradyzoites in vitro, various methods were employed such as altering the pH, addition of heat shock proteins, mitochondrial inhibitors or nitric oxide to the medium (39-41). In our survey, cyst-like structures containing the organism stained with PAS were seen that may be due to a change in pH of cell culture media. PAS stains amyllopectin granules and the presence of amyllopectin granules in bradyzoites, rarely seen in tachyzoites, further supports the difference in consumption and metabolism of the carbohydrates (42). In our study, these organisms that were cultured in a particular condition were not resistant to acid-pepsin and the resistance to acid-pepsin explains this difference. However, some researchers presented opposite reports that oral infectivity in mice or acid-pepsin digestion cannot be considered as a crucial criterion (43, 44).

The results of numerous studies on tissue culture showed that bradyzoites spontaneously were converted into tachyzoites. The rate of conversion of bradyzoites into tachyzoites appears to be strain dependent (36, 45, 46). The high spontaneous rate of cyst formation in cell culture was due to the low virulence of the strains such as type II strain (39).

Isolation of T. gondii tissue cysts and bradyzoites from the cell cultures were undertaken employing few methods such as homogenization of the tissue, bradyzoite-specific antibody titers (39, 47), and isoenzyme patterns of glycolytic and tricarboxylic acid (TCA) cycle enzymes (48-51). Using molecular biology techniques, two forms of the parasite were discriminated due to expression of particular proteins such as surface antigens (52). For instance, mRNA expression of tachyzoite-specific SRS (SAG1-related sequences) was recognized in cyst forming strains due to immunesuppression in the infected mice (53).

Although Selseleh et al. could detect bradyzoite-specific surface antigens of BAG1 mRNA in the brain of mice infected by RH strain of the parasite and treated with sulfadiazine; they were not able to find any cyst in the mice brain by microscopic observation (54).

Conclusion

This study confirmed that the RH strain of T. gondii has lost the potential for production of bradyzoite and tissue cyst forming in rats and bradyzoites cultivated in cell culture lost the most important criterion; the resistance to acidic condition, so this strain can be a candidate for future vaccine researches.

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References

1. Smith JE, Reduck NR. Toxoplasma gondii Strain Variation and Pathogenicity. In: Cary JW, Linz JE, Bhatnaga B (eds.), Microbial Foodborne Diseases: Mechanisms of Pathogenesis and Toxin Synthesis. Lancaster: Technnomic Publishing; 2000.
2. Clementino MM, Souza MF, Andrade Neto VF. Seroprevalence and Toxoplasma gondii-IgG
avidity in sheep from Lajes, Brazil. Vet Parasitol. 2007; 146: 199-203.
3. Jittapalapong S, Nimsupan B, Pinyopanuwat N, Chimnoi W, Kabeya H, Maruyama S. Seroprevalence of *Toxoplasma gondii* antibodies in stray cats and dogs in the Bangkok metropolitan area, Thailand. Vet Parasitol. 2007; 145: 138-141.
4. Garcia JL, Navarro IT, Vidotto O, Gennari SM, Machado RZ, da Luz Pereira AB, Sinhorini IL. *Toxoplasma gondii* comparison of a rhoptry-ELISA with IFAT and MAT for antibody detection in sera of experimentally infected pigs. Exp Parasitol. 2006; 113: 100-105.
5. Gilot-Fromont F, Aubert D, Belkilani S, Hermitte P, Gibout O, Geers R, Villena I. Landscape, herd management and within-herd seroprevalence of *Toxoplasma gondii* in beef cattle herds from Champagne-Ardenne, France. Vet Parasitol. 2009; 161: 36-40.
6. Dubey JP. Advances in the life cycle of *Toxoplasma gondii*. Int J Parasitol. 1998; 28: 1019-1024.
7. Tenter AM, Heckerot AR, Weiss LM. *Toxoplasma gondii* from animals to humans. Int J Parasitol. 2000; 30: 1217-1258.
8. Powell CC, Brewer M, Lappin MR. Detection of *Toxoplasma gondii* in the milk of experimentally infected lactating cats. Vet Parasitol. 2001; 102: 29-33.
9. Subauste C. Animal models for *Toxoplasma gondii* infection. Cur Protocols Immunol. 2012; 96 (19): 1-23.
10. Zenner L, Estaquier J, Darcy F, Maes P, Capron A, Cesbron-Delauw MF. Protective immunity in the rat model of congenital toxoplasmosis and the potential of excreted-secreted antigens as vaccine components. Parasite Immunol. 1999; 21: 261-272.
11. Ashburn D, Evans R, Chatterton JM, Joss AW, Ho-Yen DO. Strategies for detecting *Toxoplasma* immunity. Br J Biomed Sci. 2003; 60: 105-108.
12. Ashburn D, Chatterton JM, Evans R, Joss AW, Ho-Yen DO. Success in the *Toxoplasma* dye test. J Infec. 2001; 42: 16-19.
13. Rodgers L, Wang X, Wen X, Dunford B, Miller R, Suzuki Y. Strains of *Toxoplasma gondii* used for tachyzoite antigens to stimulate spleen cells of infected mice in vitro affect cytokine responses of the cells in the culture. Parasitol Res. 2005; 97: 332-335.
14. Sabin AB. Toxoplasmic encephalitis in children. J Am Med Assoc. 1941; 116: 801-807.
15. Hughes HP, Hudson L, Fleck DG. In vitro culture of *Toxoplasma gondii* in primary and established cell lines. Int J Parasitol. 1986; 16: 317-322.
16. Sibley LD, Boothroyd JC. Virulent strains of *Toxoplasma gondii* comprise a single clonal lineage. Nature. 1992; 359: 82-85.
17. Kreier JP. Parasitic Protozoa. New York: Academic Press. 1977.
18. Frenkel JK, Dubey JP, Hoff RL. Loss of stages after continuous passage of *Toxoplasma gondii* and Besnoitia jellisoni. J Protozool. 1976; 23: 421-424.
19. Villard O, Candolfi E, Ferguson DJ, Marcellin L, Kien T. Loss of oral infectivity of tissue cysts of *Toxoplasma gondii* RH strain to outbred Swiss Webster mice. Int J Parasitol. 1997; 27: 1555-1559.
20. Weiss LM, Kim K. The development and biology of bradyzoites of *Toxoplasma gondii*. Front Biosci. 2000; 5: D391-405.
21. Ferreira-da-Silva MF, Rodrigues RM, Ferreira de Andrade E, de Carvalho L, Gross U, Lüder CGK, Barbosa HS. Spontaneous stage differentiation of mouse-virulent *Toxoplasma gondii* RH parasites in skeletal muscle cells: an ultrastructural evaluation. Mem Do Osw Cru. 2009; 104: 196-200.
22. Dubey JP, Shen SK, Kwok OC, Frenkel JK. Infection and immunity with the RH strain of *Toxoplasma gondii* in rats and mice. J Parasitol. 1999; 85: 657-662.
23. Djurkovic-Djakovic O, Nikolic A, Bobic B, Klun I, Aleksic A. Stage conversion of *Toxoplasma gondii* RH parasites in mice by treatment with atovaquone and pyrrolidine dithiocarbamate. Microb. Infect / Institut Pasteur. 2005; 7: 49-54.
24. Zare F, Dalimi-Asl A, Ghaffarifar F. Detection of active *Toxoplasma gondii* (RH strain) in the different body tissues of experimentally infected rats. Modares J Med Sci. 2007; 9: 19-23.
25. Abdoli A, Dalimi A, Movahedin M. Impaired reproductive function of male rats infected
with *Toxoplasma gondii*. Andrologia. 2012; 44: 679-687.

26. Akins CK, Panicker S, Cunningham CL. Laboratory Animals in Research and Teaching: Ethics, Care, and Methods. Washington DC: APA Press: 2004.

27. Dubey JP, Desmons G. 1987. Serological responses of equids fed with *Toxoplasma gondii* oocysts. Equine Vet J. 48: 1239-1243.

28. Jones CD, Okhavi N, Adamson P, Tasker S, Lightman S. Comparison of PCR detection methods for B1, P30, and 18S rDNA genes of *T. gondii* in aqueous humor. Invest Ophthalmol Vis Sci. 2000; 41: 634-644.

29. Dubey JP. Refinement of pepsin digestion method for isolation of *Toxoplasma gondii* from infected tissues. Vet Parasitol. 1998; 74:75-77.

30. Dubey JP, Navarro IT, Sreekumar C, Dahl E, Freire RI, Kawabata HH, Vianna M. C, Kwok OC, Shen SK, Thulliez P, Lehmann T. *Toxoplasma gondii* infections in cats from Parana, Brazil: seroprevalence, tissue distribution, and biologic and genetic characterization of isolates. J Parasitol. 2004; 90: 721-726.

31. Dubey JP, Beattie CP. Toxoplasmosis of Animals and Man., Boca Raton FL; CRC Press: 1988.

32. Hellbrugge TF, Dahme E, Hellbrugge FK. [Experimental animal observations on transplacental infection with *Toxoplasma*]. Zeitschrift Tropenmedizin Parasitologie. 1953; 4: 312-322.

33. Hellbrugge T, Spiegler W, Grewing W. [Clinical, morphologic and serologic findings in generalized toxoplasmosis in rats]. Zentralblatt für Bakteriologie, Parastenkrankheiten und Hygiene. 1. Abt. Medizinisch-hygienische Bakteriologie, Virus-forschung Parasiologie Original. 1956; 165: 495-506.

34. Benedetto N, Folgore A, Ferrara C, Galdiero M. Susceptibility to toxoplasmosis: correlation between macrophage function, brain cyst formation and mortality in rats. New Microbiol. 1996; 19: 47-58.

35. De Champs C, Imbert-Bernard C, Belmeguenai A, Ricard J, Pelloux H, Brambilla E, Ambroise-Thomas P. *Toxoplasma gondii* in vivo and in vitro cystogenesis of the virulent RH strain. J Parasitol. 1997; 83, 152-155.

36. Saraei M, Shahnazi M, Esmaeili rostagh AR, Shojaee S, azhdari Zarmehri H, Safdari F. Lost of cystogenesis ability of *Toxoplasma gondii* RH strain in rat. 7th national and 2nd regional congress of parasitology and parasitic diseases in Iran. 2010; Oct 19-21: P 135.

37. Dubey JP, Frenkel JK. Toxoplasmosis of rats: a review, with considerations of their value as an animal model and their possible role in epidemiology. Vet Parasitol. 1998; 77: 1-32.

38. McLeod R, Frenkel JK, Estes RG, Mack DG, Eisenhauer PB, Gibori G. Subcutaneous and intestinal vaccination with tachyzoites of *Toxoplasma gondii* and acquisition of immunity to peroral and congenital *Toxoplasma* challenge. J Immunol. 1988; 140: 1632-1637.

39. Soete M, Camus D, Dubremetz JF. Experimental induction of bradyzoite-specific antigen expression and cyst formation by the RH strain of *Toxoplasma gondii* in vitro. Exp Parasitol. 1994; 78: 361-370.

40. Weiss LM, Laplace D, Takvorian PM, Tanowitz HB, Cali A, Wittner M. A cell culture system for study of the development of *T. gondii* bradyzoites. J Eukar Microbiol. 1995; 42: 150-157.

41. Lyons RE, McLeod R, Roberts CW. *Toxoplasma gondii* tachyzoite-bradyzoite interconverion. Trends Parasitol. 2002; 18: 198-201.

42. Guimaraes EV, de Carvalho L, Barbosa HS. An alternative technique to reveal polysaccharides in *Toxoplasma gondii* tissue cysts. Mem Do Institut Osw Cru 2003; 98: 915-917.

43. Dubey JP. Re-examination of resistance of *Toxoplasma gondii* tachyzoites and bradyzoites to pepsin and trypsin digestion. Parasitol. 1998; 116: 43-50.

44. Asgari Q, Mehrbani D, Motazedian MH, Kalantari M, Nouroozi J, Adnani Sadati SJ. The Viability and Infectivity of *Toxoplasma gondii* Tachyzoites in Dairy Products undergoing Food Processing. Asian J Anim Sci. 2011; 5: 202-207.

45. Lane A, Soete M, Dubremetz JF, Smith JE. *Toxoplasma gondii* appearance of specific markers during the development of tissue cysts in vitro. Parasitol Res. 1996; 82: 340-346.

46. Pippel I, Gold MC, Booth KS. Quantification of *Toxoplasma gondii* bradyzoites. J Parasitol. 1996; 82: 330-332.

47. Bohne W, Heesemann J, Gross U. Induction of bradyzoite-specific *Toxoplasma gondii* anti-
48. Ferguson DJ, Hutchison WM. An ultrastructural study of the early development and tissue cyst formation of *Toxoplasma gondii* in the brains of mice. Parasitol Res. 1987; 73: 483-491.

49. Yang S, Parmley SF. *Toxoplasma gondii* expresses two distinct lactate dehydrogenase homologous genes during its life cycle in intermediate hosts. Gene. 1997; 184: 1-12.

50. Dzierszinski F, Mortuaire M, Dendouga N, Popescu O, Tomavo S. Differential expression of two plant-like enolases with distinct enzymatic and antigenic properties during stage conversion of the protozoan parasite *Toxoplasma gondii*. J Mol Biol. 2001; 309: 1017-1027.

51. Dzierszinski F, Popescu O, Tourtel C, Slomianny C, Yahiaoui B, Tomavo S. The protozoan parasite *Toxoplasma gondii* expresses two functional plant-like glycolytic enzymes. Implications for evolutionary origin of apicomplexans. J Biol Chem. 1999; 274: 24888-24895.

52. Lekutis C, Ferguson DJ, Boothroyd JC. *Toxoplasma gondii* identification of a developmentally regulated family of genes related to SAG2. Exp Parasitol. 2000; 96: 89-96.

53. Selseleh M, Modarressi MH, Mohebali M, Shojaei S, Esragian MR, Azizi E, Keshavarz H. Real-Time RT-PCR on SAG1 and BAG1 gene expression during stage conversion in immunosuppressed mice infected with *Toxoplasma gondii* Tehran Strain. Korean J Parasitol. 2012; 50: 199-205.

54. Selseleh M, Modarressi MH, Mohebali M, Shojaei S, Esragian MR, Selseleh M, Azizi E, Keshavarz H. Real-Time RT-PCR on SAG1 and BAG1 Gene Expression during Stage Conversion in Immunosuppressed Mice Infected with *Toxoplasma gondii* Tehran Strain. Korean J Parasitol. 2012; 50(3): 199–205.