Use of chicken embryonated eggs for evaluating the virulence of *Toxoplasma gondii*

A. Setasimy · M. Namavari

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Abstract The objective of the present study was to examine the virulence of *Toxoplasma gondii* RH strain tachyzoites in embryonated eggs after continuous passage in Vero cell line. The first *T. gondii* tachyzoites was considered low passage (no passage) and then passaged for 80 times on Vero as high passage. Groups of 9-day-old embryonated eggs with ten eggs in each group were inoculated with 10^2 , 10^3 and 10^4 of low or high-passage *T. gondii* tachyzoites, and any mortality was recorded. Suitable samples from different tissues (liver, heart, and brain) of the dead embryos were collected for histopathological study. In this study, the mortality in group 10^3 and 10^4 was observed, but there was no significant differences in mortality rate in *T. gondii* low passage and high passage. This finding exactly correspond to previous studies that were performed in mice as animal model for *T. gondii* RH strain. Thus on base of this study we could introduce the embryonated eggs as an appropriate animal model to evaluate the virulence of *T. gondii* tachyzoites.

Keywords *Toxoplasma gondii* · Chicken embryonated egg · Virulence · Continuous passage

Introduction

Toxoplasma gondii is a coccidial and obligate intracellular parasite that infecting all warm-blooded animals, including

humans (Dubey 2004). If toxoplasmosis acquired in pregnant women, it can cause various congenital infections in the child (Evengård et al. 1999). Cats are the only definitive host and thus the only source of infective oocysts (Ruiz et al. 1973; Dubey 1994), vaccine development to prevent feline oocyst shedding is on progress, mostly with live vaccines, some strain of *T. gondii* are live vaccine that developed for use in sheep (Verma and Khanna 2013). The RH strain of *T. gondii* is highly virulent and produces lethal infection (Sibley and Boothroyd 1992). Attenuated, temperature-sensitive *T. gondii* strain ts-4 mutant is a suitable candidate for vaccine in order of attenuated virulence (Donald et al. 1996); however, attenuated *Toxoplasma* has not been done on Vero (African green monkey kidney) cell line. *T. gondii* can successfully propagated in the embryonated egg and uniform mortality had been observed in many studies (Que et al. 2004; Mello and Deane 1976). Recently, some researchers introduced the embryonated eggs as an animal model for experimental *Neospora caninum* infection (Khordadmehr et al. 2013; Mansourian et al. 2009; Khodakaram-Tafti et al. 2012) and also for evaluating the virulence of *N. caninum* tachyzoites (Namavari et al. 2012). Since *T. gondii* and *N. caninum* are closely related parasites of the phylum Apicomplexa via the comparative genomic (Reid et al. 2012), the aim of this study was to evaluate *T. gondii* virulence by high passage and low passage in Vero cell line using embryonated egg as an animal model.

Materials and methods

In this investigation, Vero cell lines and *T. gondii* RH strain were obtained from the Razi Vaccine and Serum Research Institute, Karaj, Iran. Vero cells were maintained in at

A. Setasimy
Islamic Azad University Fars Science and Research Branch,
Shiraz, Iran

M. Namavari (✉)
Razi Vaccine and Serum Research Institute, Shiraz, Iran
e-mail: namavari@yahoo.com; m.namavari@rvsri.ac.ir

37 °C in an atmosphere of 95 % air and 5 % CO₂. The tachyzoites of *T. gondii* were cultured on a 24-hour-old monolayer of Vero cell line and maintained in DMEM Medium (Sigma Co., USA) supplemented with 2 % fetal calf serum, penicillin/ml (10,000 U), streptomycin/ml (100 µg) and streptomycin (25 µg) (Invitrogen, USA) at 37 °C with 5 % CO₂ (Hemphill et al. 1996). Tachyzoites were harvested from infected cell cultures when about 80–90 % of the Vero host cells had lysed, as determined by microscopic examination of monolayers for cytopathic effects (CPE), and then counted using a hemacytometer. Viability of the partially purified tachyzoites was evaluated by trypan blue (Gibco) exclusion test (Chamberland and Current 1990). The first *T. gondii* was considered as low passage (no passage) and then passaged for 80 times in vitro (on Vero) as high passage.

Groups of 9-day-old embryonated eggs with ten eggs in each group were inoculated with 10², 10³ and 10⁴ of low-passage or high-passage of *T. gondii* tachyzoites on Vero cell line and any mortality was recorded. Suitable samples from various tissues (liver, heart, and brain) of the dead embryos were collected for histopathological and molecular studies. Samples for histopathological study were fixed in 10 % buffer formalin, processed regularly, and embedded in paraffin. Paraffin blocks were then sectioned at about 5 µm, stained with hematoxylin and eosin (H&E staining), and studied microscopically with a light microscope.

DNA extraction from 20 to 40 mg of tissues of liver, heart and brain have done using the DNP kit (sina gene co., Iran), according to the manufacturer's instructions. For the detection of parasite DNA, NP6/NP21 primers was used (Burg et al. 1989).

Results

In the group with 10³ and 10⁴ low passage tachyzoites, eight and ten embryonated eggs died, respectively. In the groups with 10³ and 10⁴ high passage tachyzoites, five and seven embryonated eggs died, respectively (Fig. 1). In groups with 10² low passages and either high passage, no mortality was observed. Microscopic examination revealed acute toxoplasmosis in the dead embryos. In the cases with 10² tachyzoites, no inflammations were observed in any groups with high or low passage. In other groups granulomatous inflammation was seen with aggregation of numerous macrophages in the liver and heart. In all the point of histopathological changes no differences were seen in different groups of low and high passages. Molecular assay showed the *T. gondii* DNA the embryo liver inoculated with the low and high passage parasite, like in the heart. No parasite DNA were detected in the brain of the dead embryos in any group.

Fig. 1 The mortality rate of chicken embryos inoculated with low and high passage tachyzoite of *T. gondii* RH strain 103 and 104 inoculum doses

Statistical analysis

For statistical analysis the Fisher's exact test was used. There was no significant difference between the mortality rate of the low and high passage in all doses of the infected groups with *T. gondii* tachyzoites ($p < 0.05$).

Discussion

The development of defensive immunity to *T. gondii* following natural infection in many host species has led researchers to look at vaccination as a strategy to control disease (Innes et al. 2009). Immunization with live, attenuated *T. gondii* parasites devolved better protection compared to inactivated tachyzoites (Waldeland and Frenkel 1983). Attenuation of parasite virulence has been effectively applied in the development of live vaccines against some protozoan parasites like *N. caninum* (Namavari et al. 2012); *Babesia bovis* and *Babesia bigemina* (Pipano et al. 2002). Attenuation of *T. gondii* virulence has been utilized like the S48 which was maintained in the laboratory by repeated passages in mice (Wilkins et al. 1988); TS-4 isolate which is a chemical mutagenesis of the RH strain resulted in the temperature sensitive strain (Pfefferkorn and Pfefferkorn 1976).

MacFarlane and Ruchman (1948), Warren and Russ (1948) had introduced the embryonated egg as a good animal model which could enhance successfully and presented appropriate antigen which fixed complement with *Toxoplasma* immune animal serum.

Besides the availability and affordability of embryonated eggs, the most important reason is the need for a very low LD50 dose of *T. gondii* for infection in the embryonated chicken egg and less incubation time in comparison to the mice. In this study the process of attenuation *T. gondii* virulence was performed by the continuous passage of parasites in the cell culture, which have not lead to

reduction in virulence. The results of this study indicated course of infection in embryonated egg with *T. gondii* RH strain was independent on the number of passage; therefore the embryonated eggs inoculated with the high-passage tachyzoites compared to those receiving the low-passage tachyzoites have the same mortality rate.

Wilkins et al. (1988) demonstrated *T. gondii* S48 strain became an attenuated strain following long ($\times 3,000$) passages in vivo condition. Immunization with attenuated strain reduced neonatal mortality in mice (Buxton et al. 1991); however, Chamberland and Current (1990), showed that continuous passage of *T. gondii* RH grown in mouse macrophage cell lines (in vitro condition) retained virulence, which is confirming with our finding. We conclude on base of this investigation embryonated egg could be a suitable animal model to evaluating the virulence of *T. gondii*.

Data from previous studies shown that biological and genetical differences between strains of *T. gondii*. As *T. gondii* S48 strain became an attenuated strain following serial passages, in contrast passage in cell-culture over a several months did not affect the pathogenicity of *T. gondii* RH strain in mouse model. In conclusion here we reconfirmed this finding by use of embryonated chicken egg. Consequently we could introduce embryonated chicken egg as suitable animal model to assessment of pathogenicity of different strains of *T. gondii*.

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