Viability and infectivity analysis of *Toxoplasma gondii* under axenic conditions

Análise da viabilidade e infectividade de taquizoítas de Toxoplasma gondii em condições axênicas

Marialice da Fonseca Ferreira-da-Silva[1,3], Andrea Henriques Pons[2], Roberto Carlos Tedesco[3], Helene Santos Barbosa[4]

[1] PhD in Cellular and Molecular Biology, Instituto Oswaldo Cruz-Fundação Oswaldo Cruz (Fiocruz), Rio de Janeiro, Brazil. Postdoctoral fellow in the Department of Cell Genetics, University of Cologne, Germany.
[2] PhD, Associate Research at Laboratory for Innovations in Therapy, Education and Bioproducts, Instituto Oswaldo Cruz-Fiocruz, Rio de Janeiro, Brazil.
[3] PhD, Research at Structural Biology Laboratory.Instituto Oswaldo Cruz-Fiocruz, Rio de Janeiro, Brazil.
[4] PhD, Senior Researcher and Head of the Structural Biology Laboratory at Instituto Oswaldo Cruz-Fiocruz, Rio de Janeiro, Brazil.

ABSTRACT

Aims: To investigate the infectivity and viability of the tachyzoite form of *Toxoplasma gondii* maintained in axenic medium. **Methods:** Tachyzoites of *Toxoplasma gondii* isolated from infected mice were suspended in phosphate-buffered saline pH 7.2 or phosphate-buffered saline pH 7.2 plus 10% or 20% foetal calf serum, and incubated for 24 and for 48 hours at 37ºC. Afterwards the parasites were: (i) incubated with propidium iodide and analysed by flow cytometry, using the fluorescence-activated cell sorting (FACS) system; (ii) injected in mice to check the parasite viability in axenic conditions and, (iii) added to mouse embryonic fibroblasts to investigate their infectivity and ability to intracellular development. **Results:** Analysis by flow cytometry showed that *Toxoplasma gondii* tachyzoites maintained in phosphate-buffered saline supplemented with 20% foetal calf serum displayed high cellular viability. The parasites kept their infectivity in both the *in vivo* and the *in vitro* systems, respectively demonstrated by their ability to replicate in mice and to form rosettes in mouse embryonic fibroblasts. **Conclusions:** Our data open new perspectives for the study of different aspects of *Toxoplasma gondii* cell biology, including nutrition mechanisms, *in vitro* drug trials, or cellular and molecular studies to be performed directly on the parasite.

Keywords: *Toxoplasma gondii*/infectivity; *TOXOPLASMA*/isolation & purification; FLOW CYTOMETRY; MICROBIAL VIABILITY.

RESUMO

Objetivos: investigar a infectividade e a viabilidade de formas taquizoítas de *Toxoplasma gondii* mantidas em meio axênico. **Métodos:** taquizoítos de *Toxoplasma gondii* foram isolados de camundongos infectados, ressuspensos em tampão fosfato-salino pH 7,2 ou tampão fosfato-salino pH 7,2 mais soro fetal bovino a 10% ou 20% e incubados por 24 e 48 horas a 37ºC em atmosfera contendo 5% de CO_2. A seguir os parasitos foram: (i) incubados com iodeto de propídio e analisados por citometria de fluxo utilizando a técnica de separação de células ativada por fluorescência (*fluorescence activated cell sorting* – FACS); (ii) injetados em camundongos para analisar a infectividade dos parasitos mantidos em condições axênicas; e (iii) adicionados à cultura de fibroblastos de embrião de camundongo para investigar a sua capacidade infectiva e multiplicativa com formação de rosetas e lise celular. **Resultados:** a análise por citometria de fluxo mostrou que os taquizoítos de *Toxoplasma gondii* mantidos em tampão fosfato-salino suplementado com 20% de soro fetal bovino apresentaram elevada viabilidade celular. Os parasitos mantiveram sua infectividade nos sistemas *in vivo* e in *vitro*, respectivamente demonstrada por sua capacidade de se replicar em camundongos e de infectar culturas de fibroblastos. **Conclusões:** nossos dados abrem novas perspectivas para o estudo de diferentes aspectos da biologia do *Toxoplasma gondii*, incluindo mecanismos de nutrição, ensaios experimentais de drogas *in vitro*, ou estudos da biologia celular e molecular diretamente sobre o parasito.

Descritores: *Toxoplasma gondii*/infectividade; *TOXOPLASMA*/isolamento & purificação; CITOMETRIA DE FLUXO; VIABILIDADE MICROBIANA.

Endereço para correspondência/Corresponding Author:
HELENE SANTOS BARBOSA
Laboratório de Biologia Estrutural, Instituto Oswaldo Cruz, Fiocruz
Av. Brasil 4365
CEP 21040-900, Rio de Janeiro, RJ, Brasil
Phone/Fax 55 21 2260-4434
E-mail: helene@ioc.fiocruz.br

INTRODUCTION

Toxoplasma gondii (*T. gondii*) is a highly complex unicellular parasite, which causes severe systemic disease in immunocompromised individuals, and congenital disease leading to hydrocephalus and mental retardation in children.[1-3] As an obligate intracellular parasite, *T. gondii* must invade a host cell in order to survive and replicate. This requirement limits the experimental procedures performed in laboratories, because the parasites must be maintained in cell cultures or animal models. The inability to cultivate *T. gondii in vitro* in the absence of a host cell is due to the need of a compartment protecting the parasite from the host immune response, and also providing cholesterol, purines and other essential factors for its intracellular lifecycle.[4,5] The flow of extracellular molecules from the medium to the intracellular parasite faces barriers composed by three membranes – the host cell´s, the parasitophorous vacuole´s and that of the parasite. Each membrane constitutes a potentially permeable barrier that must be either crossed or bypassed.[6] Currently, the maintenance of tachyzoite forms is restricted to the infection in mice and/or in cell culture.[7,8] In the literature there are only few reports on experimental protocols aiming the *in vitro* maintenance of *T. gondii*, in either defined or complex medium.[9] In the present report, we established an *in vitro* protocol for the maintenance of *T. gondii* tachyzoites in axenic medium for 24 h at 37ºC in CO_2 atmosphere, evaluated by the parasite viability and infectivity in both *in vivo* and *in vitro* models.

METHODS

All procedures were carried out in accordance with the guidelines established by the Brazilian College of Animal Experimentation (COBEA, *Colégio Brasileiro de Experimentação Animal*), by the Committee of Ethics for the Use of Animals of Fundação Oswaldo Cruz (CEUA, *Comissão de Ética no Uso de Animais*-Fiocruz), license 0229-04, and by the National Advisory Committee for Laboratory Animal Research (NACLAR) Guidelines on the Care and Use of Animals for Experimental Purposes and Infectious Agents.

Parasites

Tachyzoites of the virulent RH strain of *T. gondii* were maintained by serial intraperitoneal passage in Swiss mice (female 18-20 g) purchased from Creation of Laboratory Animals Center (CECAL, *Centro de Criação de Animais de Laboratório*), Fiocruz, Brazil. After 48-72 h of 10^5 parasites inoculation in mice, the exudates from several mice were harvested in phosphate-buffered saline pH 7.2 (PBS) and centrifuged for 7 to 10 min at 200 g at room temperature to remove peritoneal cells and cellular debris. The supernatant enriched with the parasites was collected and again centrifuged for 10 min at 1000 g. The parasites recovered from the pellet were then washed with PBS and utilized in the experiments.

Axenic medium

Tachyzoites isolated from infected mice were suspended in the following axenic media: (a) PBS; (b) PBS with addition of 10% foetal calf serum (FCS); (c) PBS with addition of 20% FCS and incubated for 24 and for 48 hours at 37ºC in CO_2 atmosphere. Direct counts of parasites were carried out daily in a Neubauer chamber. Each experiment was repeated at least three times.

Toxoplasma gondii viability

After the different protocols above described, 10^6-10^7 tachyzoites were collected in Eppendorf tubes and incubated for 30 min at 4°C with 50 µg/ml propidium iodide (PI) (Molecular Probes). After incubation, the parasites were kept on ice until analysis. Data acquisition and analysis were performed by fluorescence activated cell sorting (FACS), using the FACSCalibur flow cytometer (Becton- Dickinson, San Jose, USA) equipped with the Cell Quest software (Joseph Trotter, Scripps Research Institute, San Diego, USA). A total of 10,000 events were acquired in the region previously established as that corresponding to the parasites. Positive controls for PI staining were obtained by incubating parasites in the presence of 0.2% saponin.

In vivo infectivity studies

Parasites maintained in PBS supplemented by 20% FCS for 24 h at 37ºC in CO_2 atmosphere were washed and inoculated intraperitoneally with 10^5 parasites/animal in 36 Swiss male mice. After three days of infection, the parasitemia was quantified by counting free tachyzoites in the peritoneal exudate of the animals using in a Neubauer chamber.

In vitro infectivity studies

Mouse embryonic fibroblasts cultures were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% FCS, non essential aminoacids, 2 mM *L*-glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin and 100 mg/ml streptomycin. The monolayers were cultivated for 24 h at 37ºC in a 5% CO_2 atmosphere, and then used for the experiments. *T.*

gondii tachyzoites maintained in PBS plus 20% FCS for 24 h at 37ºC in CO_2 atmosphere were washed and inoculated (1:1 parasites:host cell ratio) in fibroblasts cultures. After 24 and 48 h of interaction, the cultures were fixed for 5 min at room temperature in 4% paraformaldehyde diluted in PBS, then washed three times (10 min each) with PBS. All results were based on three independent experiments performed in duplicate. The cells were observed under a Zeiss Axiovert 200 microscope (Carl Zeiss, Germany) and the images were obtained with a CoolSNAP camera (Roper Scientific Inc., Evry, France).

RESULTS

Flow cytometry assays were performed using PI incorporation, which could provide quantitative analysis for the parasite viability. The positive controls were fresh parasites previously incubated with 0.2% of saponin. Only 4% of the parasites after saponin treatment displayed membrane integrity, as opposed to 99% of the untreated ones. The PI incorporation assays showed that the parasites maintained for 24 h in PBS supplemented with 10 and 20% FCS displayed 88.5 and 90% of viability, respectively. However, after 48 h of incubation, the cellular viability largely decreased independently of the serum concentration (Figure 1).

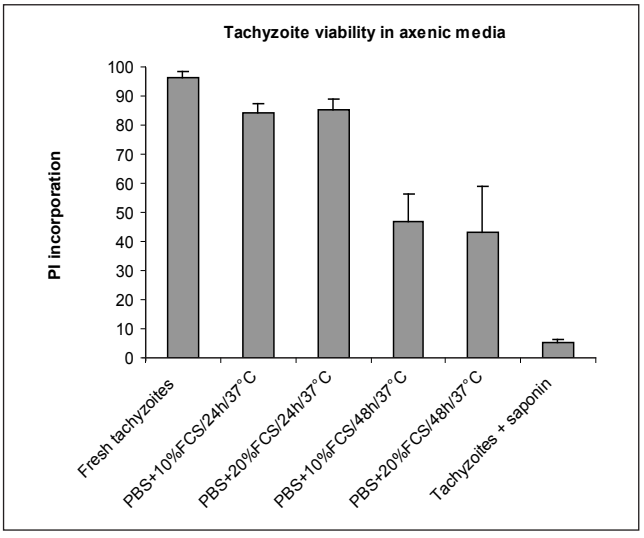

Figure 1. Parasite viability tested by propidium iodide (PI) incorporation. Parasites maintained in phosphate-buffered saline pH 7.2 (PBS) + 10% or 20% foetal calf serum (FCS) for 24 and 48h, at 37ºC in CO_2 atmosphere, were incubated with propidium iodide and analysed by flow cytometry using the fluorescence-activated cell sorting method. The control groups were constituted by fresh recently released parasites and parasites incubated with 0.2% saponin.

Figure 2 shows a representative experiment of the cellular viability of parasites maintained for 24 and 48 h in PBS+20% FCS monitored by PI labelling. The parasites were seen in clusters by phase contrast microscope (Figure 3A) and only a reduced number of parasites presented positive labelling for PI (Figure 3B), confirming the data obtained by flow cytometry.

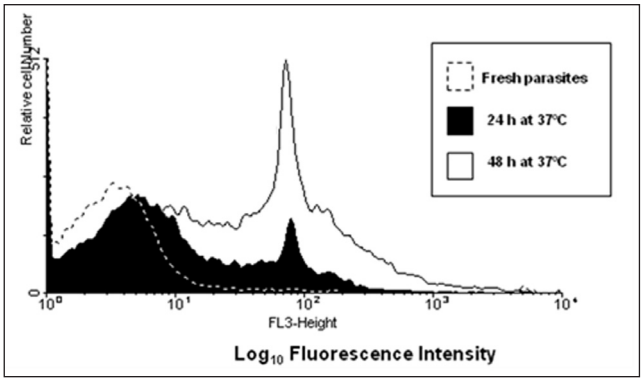

Figure 2. Overlay obtained by fluorescence-activated cell sorting (FACS) system of flow cytometry, of parasites maintained in axenic conditions (phosphate-buffered saline pH 7.2 + 20% foetal calf serum for 24 and 48h at 37ºC) and CO_2 atmosphere and incubated with propidium iodide. Fresh parasites showed 96% of viability and tachyzoites maintained for 24 and 48 h in axenic conditions presented 82% and 49% of membrane integrity, respectively.

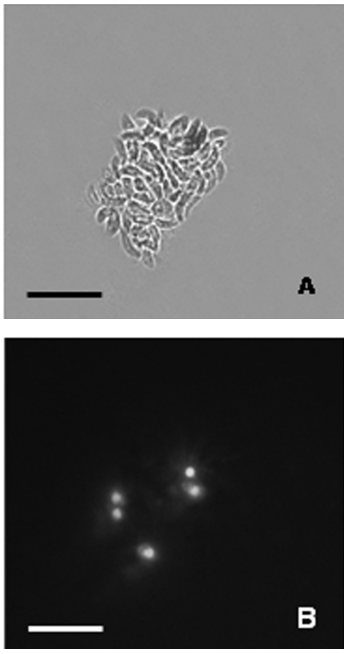

Figure 3. Tachyzoites of *T. gondii* maintained in phosphate-buffered saline pH 7.2 + 20% foetal calf serum at 37ºC and CO_2 atmosphere and stained with propidium iodide. **A)** Parasites observed by phase contrast microscopy. **B)** Analysis by fluorescence microscopy. Most of the parasites were not labelled, demonstrating high cellular viability. Bar: 10 μm.

In vivo assays

This study showed that mice infected with parasites submitted to the different experimental protocols as above described presented the typical characteristics of *T. gondii* infection including hispid hair, low physical activity and apathy. At the day 3 post-infection, 35 out 36 inoculated mice presented free tachyzoites in the peritoneal exudate, resulting in 97.2% of positive mice. The percent of cumulative mortality was 27.7, 55.5 and 83.4% at the 4, 5-6 and 7-8 post-infection days, respectively (data not shown).

In vitro assays

The proliferation ability of tachyzoites maintained for 24 h in PBS+20% FCS was demonstrated by their ability of infecting mouse embryonic fibroblast cultures (Figure 4). After 24 of interaction several parasitophorous vacuoles were observed containing tachyzoites in division by endodyogeny (Figure 4A). Most parasitophorous vacuoles contained groups of tachyzoites randomly arranged in rosettes due to synchronous division (Figures 4B and 4C).

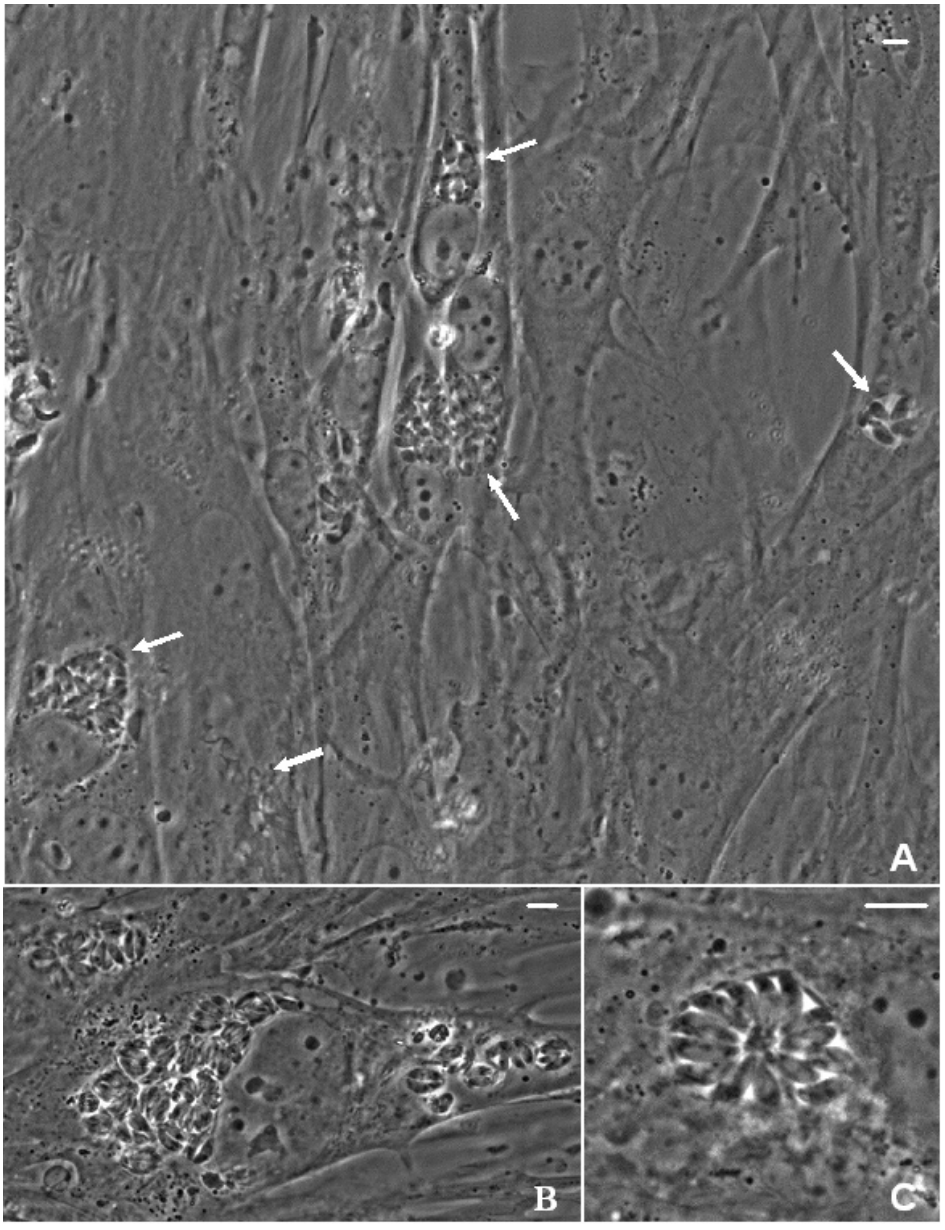

Figure 4. *In vitro* infectivity and rosettes formation of tachyzoites from *T. gondii* RH strain previoulsy maintained in phosphate-buffered saline pH 7.2+20% foetal calf serum at 37ºC in CO_2 atmosphere for 24 h. **A)** Parasitophorous vacuoles containing several tachyzoites in division by endodyogeny (arrows). **B-C)** Parasitophorous vacuoles containing groups of tachyzoites randomly arranged in rosettes due to synchronous division. Bars: 5 µm.

DISCUSSION

This paper described that *T. gondii* tachyzoites could be maintained in axenic medium using a simple and routine laboratory solution (PBS + 20% FCS), namely axenic medium, up to 24 h, without loss of parasite infectivity and viability in both the *in vivo* and the *in vitro* systems. This approach represents a powerful tool, which can be used in the investigation of *T. gondii* biology, including nutrition mechanisms, *in vitro* drug trials, or cellular and molecular studies to be performed directly on the parasite itself without the necessity of host cell interaction. The previous attempts performed by other groups failed to find a suitable synthetic medium for the *in vitro* maintenance of *T. gondii*.[10,11] There is an increasing interest about *T. gondii* biology, in order to established growth factors or other molecules required for parasite multiplication in axenic media.[12] In the absence of a defined culture medium that could enable the extracellular multiplication of *T. gondii*, large amounts of parasites have been obtained in laboratory by employing experimental animals, mostly mice models, since they are highly sensitive to the parasite infection. An alternative method is the use of cell cultures from different origins, due to the enormous spectrum of mammalian cells that are susceptible to the *T. gondii* invasion.[13, 14]

As an obligate intracellular pathogen, *T. gondii* resides exclusively inside a parasitophorous vacuole, which membrane is molecularly modified by the parasite itself during its invasion and afterwards during its intracellular cycle.[15-17] However, this compartment represents a limiting experimental factor for the direct access to the parasite, restricting the study of many aspects of cellular biology, including the screening of effective drugs and the analysis of the internalization of nutrients by *T. gondii.*

It has been postulated 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 23-25ºC in extracellular medium.[10] On the other hand, exudates enriched with parasites or even infected tissues kept at 4ºC for 7-14 days still show some viable organisms when maintained in Tyrode solution.[18,19] In this context, it has been advisable to use fresh parasites recently harvested from infected mice to perform *in vivo* or *in vitro* studies.[10] Since the investigation of the infectivity and viability of tachyzoite forms maintained in axenic medium has been poorly explored in the literature, the development of the present work employing different methodological approaches for evaluation of these parameters is largely justified. The flow cytometry technique is a fast, simple and reproducible tool to analyze unicellular events.[20] This method confirmed that higher viability rates were obtained incubating extracellular parasites with buffered saline supplemented with 20% FCS. Our data are in agreement with experiments from other groups[10,21,22] which described that the addition of serum is necessary to keep the viability and infectivity of *T. gondii* as well as the excretion of antigens by tachyzoites.

The presence of free tachyzoites in the mouse peritoneal exudate as well as the high parasitemia and mortality rates observed in mice infected with parasites maintained in PBS supplemented with 20% FCS were similar to the control group inoculated with freshly harvested parasites. The *in vitro* infectivity was confirmed by the demonstration of the infection of fibroblast cultures (with parasites under division and rosettes formation) by parasites incubated for 24 h in PBS+20% FCS. These data allow the development of experimental protocols for the direct test of potential drugs against the parasite and their selection for further *in vivo* assays.

In our *in vitro* assays we also noted that the maintenance of tachyzoites of *T. gondii* in axenic conditions doesn't allow the parasite growth, a clearly expected finding. It has been described that *T. gondii* is already sensitive at 23ºC, and at 37ºC it rapidly dies when maintained in extracellular medium.[10] We demonstrated here that this parasite can support adverse conditions for a limited time (until 48 h at 37ºC), keeping its infectivity and cellular viability. These obtained from different approaches demonstrated the possibility to study different aspects of the cellular biology of *T. gondii*. Our study provides news perspectives in the direct approaches of drug development against the parasite, as well as the application in the studies concerning the dynamics of nutrients incorporation by tachyzoite forms, a still little explored theme.

ACKNOWLEGEMENTS

The authors thank Dr. Solande Lisboa de Castro for critically reading this manuscript. To Sandra Maria de Oliveira Souza and Marielle Moreira for their excellent technical assistance. This work was supported with grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ) and Instituto Oswaldo Cruz/Fiocruz.

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