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Short communication

In contrast to *Toxoplasma gondii*, *Neospora caninum* tachyzoites did not sustain multiplication *in vitro* at increased incubation temperatures

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ABSTRACT

Neospora caninum and *Toxoplasma gondii* are coccidian parasites that infect a wide range of mammalian and avian species. While viable *T. gondii* has been *in vitro* isolated in natural infections from wild and domestic birds, attempts to isolate *N. caninum* from naturally-infected birds were unsuccessful. We speculate that body temperatures of birds, which are usually higher than those of mammals, may impair the multiplication of *N. caninum*. In contrast to *N. caninum*, *T. gondii* can grow *in vitro* at temperatures higher than 37 °C. To test the hypothesis that *N. caninum* tachyzoites are impaired to grow *in vitro* at high temperatures, three strains of *N. caninum* (NC-1, NC-Liverpool, and NC-Bahia) and three of *T. gondii* (RH, ME-49 and NED) were cultivated at gradually increasing temperatures starting at 37 °C up to 41.5 °C. A permanent chicken cell line was chosen for the study. Parasites were observed microscopically and their presence in culture was evaluated by species-specific conventional PCRs. In a second experiment, growth rates of *T. gondii* (RH strain) and *N. caninum* (NC-1 strain) were evaluated after direct passage of tachyzoites from 37 °C to 41.5 °C, and quantified by real-time PCR. In addition to comparisons between *N. caninum* and *T. gondii*, growth rates of three *T. gondii* strains were compared at high temperatures. *Neospora caninum* tachyzoites could not sustain multiplication at temperatures between 39 °C and 41.5 °C. *Toxoplasma gondii* tachyzoites continued to multiply at the same experimental conditions. Direct passage of *N. caninum* tachyzoites from 37 °C to 41.5 °C caused a significant decrease in the number of parasites during 96 h of observation, while *T. gondii* had a significant increase in the number of stages after the same period of time. *T. gondii* RH strain (clonal type I) presented a different growth rate at 41.5 °C when compared with type II and type III strains. In conclusion, multiplication of *N. caninum* tachyzoites *in vitro* was inhibited at temperatures similar to those of chickens, what may be one of the reasons that isolation of the parasite is difficult in avian species. In contrast to *N. caninum*, *T. gondii* continued to grow at 41.5 °C.

1. Introduction

Toxoplasma gondii and *Neospora caninum* are closely related coccidian parasites that share many biological, ultrastructural and genetic characteristics (Lorenzi et al., 2016; Speer et al., 1999). Contaminations of soil and water are regarded as major sources of infection for several animal species. A number of findings suggest that in addition to *T. gondii*, *N. caninum* is also able to naturally infect mammals and birds (Costa et al., 2008; Darwich et al., 2012; Dubey, 2002; Tenter et al., 2000). *Neospora caninum* DNA and/or antibodies have been detected from naturally infected birds (Costa et al., 2008; Darwich et al., 2012; Gonçalves et al., 2012; Salant et al., 2015). However, viable *N. caninum* organisms have never been isolated from naturally-infected birds, whereas *T. gondii* has been isolated from natural infections in birds in numerous studies (Dubey, 2010).

Chickens were demonstrated to be useful sentinels for *T. gondii* contamination in soil, as these animals feed directly on the ground (da Silva et al., 2003; Dubey et al., 2002). Since *N. caninum* oocysts may also be spread on the soil, chickens were also suspected to serve as good markers for environmental contamination with *N. caninum* oocysts. In experimental infections with *N. caninum* viable tachyzoites could not be *in vitro* isolated from adult chickens (Furuta et al., 2007). In addition, dogs fed tissues from experimentally infected chickens or quails (*Coturnix coturnix japonica*)

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did not excrete *N. caninum* oocysts in their feces (de Oliveira et al., 2013; Munhoz et al., 2014). Our research group attempted to isolate *N. caninum* from seropositive chickens by bioassay in gamma-interferon knockout mice, which did not yield any isolate (Gonçalves et al., 2012). In this same study, several *T. gondii* isolates were obtained from *T. gondii*-seropositive chickens by bioassay in Swiss mice (Gonçalves et al., 2012). The difficulty to isolate *N. caninum* from avian species was attributed to several potential factors, including the elevated body temperature of birds (Gonçalves et al., 2012). Overall, birds have higher body temperatures than mammals (Prinzinger et al., 1991). The average body temperature of an adult chicken is 41.5 °C (Richards, 1971). *Toxoplasma gondii* and *N. caninum* are frequently cultured at 37 °C that corresponds to the mean body temperature of several mammals (Prinzinger et al., 1991). *Toxoplasma gondii* is able to grow *in vitro* at temperatures higher than 37 °C (Pfefferkorn and Pfefferkorn, 1976).

To test the hypothesis that *N. caninum* tachyzoites have a lower capacity of multiplication at high temperatures than *T. gondii* tachyzoites, we evaluated the *in vitro* growth of both parasites at temperatures ranging from 37 °C to 41.5 °C using a chicken cell line. In addition, we compared the growth rates of three *T. gondii* strains (representing three clonal lineages of *T. gondii*, i.e. type I, II and III) at 41.5 °C.

2. Materials and methods

2.1. Cell lines

Three permanent cell lines were chosen for this study. MARC-145 (Kim et al., 1993) and Vero cells (Yasumura and Kawakita, 1963) are both derived from monkeys' kidneys, and DF-1 cells (UMNSAH/DF-1) (Foster and Foster, 1997) are cells derived from chicken embryo. The three cell lines were grown in a humidified incubator at 37 °C containing 5% CO_2 and 95% air. The media used to maintain all cell lines were supplemented with 1% antibiotic solution (10,000 IU penicillin and 10,000 μg streptomycin/ml), 10% fetal calf serum (FCS) and non-essential amino acids. Thrice weekly (Mondays, Wednesdays and Fridays) culture media were changed. Cells were split depending on the growth rate. Culture media were previously heated in water-bath at the same temperature of the incubator before adding to culture flasks or plates.

2.2. Toxoplasma gondii *and* N. caninum *strains*

T. gondii tachyzoites of the RH (Sabin, 1941), ME49 (Lunde and Jacobs, 1983) and NED (Howe and Sibley, 1995) strains, which belong to genotypes I, II and III (Howe and Sibley, 1995), respectively, were cultivated on MARC-145 cell monolayers. The cells were maintained with Dulbecco's Modified Eagle's Medium (DMEM) supplemented as cited above. Three *N. caninum* strains, including NC-1 (Dubey et al., 1988), NC-Liverpool (Barber et al., 1995) and NC-Bahia (Gondim et al., 2001) were cultured at the same conditions described for *T. gondii*. NC-Bahia strain employed for this study had been stored in liquid nitrogen and had a history of 19 passages in cell culture after its original isolation from peritoneum cavity of a gerbil (*Meriones unguiculatus*).

2.3. Adaptation of DF-1 cells from 37 °C to 42 °C

Prior to the experiment using *T. gondii* and *N. caninum* strains in different temperatures, DF-1 cells were cultured at 37 °C for three weeks. The incubation temperature for the cell culture was increased at 1 °C every three weeks until 42 °C. The cell monolayers were daily observed for any change in morphology or attachment conditions to the culture flasks.

2.4. Cultivation of T. gondii and N. caninum strains at temperatures between 37 °C and 41.5 °C

Toxoplasma gondii (RH, ME-49 and NED) and *N. caninum* (NC-1, NC-Liverpool, and NC-Bahia) strains were initially produced in Marc-145 cells. Infected cells were scraped and the contents filtered through a 5 μm filter (Millipore, Eschborn, Germany). Purified tachyzoites of each parasite strain were counted in Neubauer chambers and inoculated in 25-cm^2 flasks (7×10^4 tachyzoites/flask) containing DF-1 cell monolayers. Two flasks were prepared for each strain totalizing 12 flasks. The infected DF-1 cells were maintained for three weeks at 37 °C. The incubation temperature was increased at 1 °C for every three weeks until 41.0 °C. After three weeks at 41.0 °C, the temperature was raised to 41.5 °C and the cells were kept for additional three weeks. Once a week, the total monolayer of a flask per strain was passed to a new flask at the same temperature. Only intracellular tachyzoites were used for each passage.

The content of the second flask was rinsed with sterile PBS and submitted to DNA extraction (section 2.7) followed by conventional PCR (section 2.8). Uninfected DF-1 cells were used as negative controls for each DNA extraction and PCR.

2.5. Growth rates of T. gondii (RH) and N. caninum (NC-1) cultured directly from 37 °C to 41.5 °C

Growth rates of *T. gondii* (RH strain) and *N. caninum* (NC-1) tachyzoites were evaluated by real-time PCR (qPCR) at 4 and 96 h post-infection (p.i.). Tachyzoites were obtained from infected MARC-145 cells cultured at 37 °C when most parasites were intracellular and the majority of host cells were intact. Infected cells were scraped and tachyzoites were purified as described in section 2.4. Purified tachyzoites were added to DF-1 monolayers in 24-well culture plates incubated at 41.5 °C. In order to form homogeneous monolayers, 3×10^4 cells were added in each well 46 h before tachyzoites were inoculated. To better determine the number of tachyzoites added in each well, the same volume as the inoculum was placed in a 1.5 ml reaction tube and measured by qPCR after DNA extraction. The amount estimated in the 1.5 ml reaction tubes was designated as "0 h" p.i. Five wells per parasite were measured in duplicate at each analyzed period. Cultures were maintained in Hamís medium supplemented with 1% antibiotics solution and non-essential amino acids. Plates were kept 96 h in culture and had their media renewed 4 and 72 h p.i. Prior to DNA extraction, media were discarded and each well was carefully rinsed thrice with 3 ml of serum-free media. Cell monolayers were then recovered in 200 μl of lysis buffer, 25 μl of proteinase K and 200 μl of another lysis buffer contained in the DNA extraction commercial kit (NucleoSpin® Tissue, Macherey and Nagel, Düren, Germany). Thereafter the contents of the wells were individually placed in 1.5 ml reaction tubes and subjected to DNA extraction.

2.6. Growth rates of three T. gondii strains at 41.5 °C

Three strains of *T. gondii* (RH, ME49 and NED) maintained at 41.5 °C had their growth evaluated by means of qPCR at 4, 24 and 72 h p.i. Six wells per strain were examined in duplicate at each time frame. Tachyzoites were added to DF-1 monolayers cultivated in 24-well culture plates. The same amount of tachyzoites deposited in each well was added to 1.5 ml reaction tubes for accurate quantification of parasite DNA and designated as "0 h p.i". Before DNA extraction from individual wells, the media were removed and the entire cell monolayer from each well had its total DNA extracted for PCR.