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Comparison of use of Vero cell line and suspension culture of murine macrophage to attenuation of virulence of *Neospora caninum*

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ABSTRACT

In this study the tachyzoite yields of *Neospora caninum* were compared in two cell lines: Vero (African Green Monkey Kidney) and suspension culture of murine macrophage (J774) cell lines. Then, *N. caninum* were continuously passaged in these cell lines for 3 months and the effect of host cells on virulence of tachyzoites was assessed by broiler chicken embryonated eggs. Inoculation was performed in the chorioallantoic (CA) liquid of the embryonated eggs with different dilutions (0.5×10^4, 1.0×10^4, 1.5×10^4) of tachtzoites isolated from these cell cultures. The mortality pattern and pathological changes of the dead embryos and hatched chickens were noted. Tissue samples of brain, liver and heart were examined by histopathological and detection of DNA of parasite by polymerase chain reaction (PCR). Also, consecutive sections of the tissues examined histologically were used for immunohistochemical (IHC) examination. Embryos inoculated with tachyzoites derived from Vero cell line (group V) showed a higher mortality rate (100%) than the embryos that received tachyzoites derived from J774 cell line (group J) (10% mortality rate). The results of this study indicated that the culture of *N. caninum* in J774 cell led to a marked increase in the number of tachyzoite yields and rapid attenuation in comparison to Vero, so the results were confirmed by IHC and PCR. This study is the first report of the significant effect of host cell on the attenuation of virulence of *N. caninum* tachyzoites. These findings could potentially provide a practical approach in the mass production of *N. caninum* tachyzoites, and also in producing live attenuated vaccine.

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1. Introduction

Neospora caninum is a cyst-forming coccidian parasite intimately related to *Toxoplasma gondii* species (Dubey, 2003). Neosporosis has emerged as a serious disease of cattle and dogs worldwide. Birds such as pigeon and chicken embryonated egg are investigated as suitable models for experimental *N. caninum* infection (Mineo et al., 2009; Furuta et al., 2007). Also, apicomplexa- like cysts were found in muscular tissues of wild and captive birds (Mineo et al., 2011).

There are currently no suitable chemotherapeutic agents to prevent transplacental transmission or to eliminate the parasite in cattle, making the development of an effective vaccine highly desirable. Several studies indicate vaccination may be a feasible option for the control of bovine neosporosis (Innes et al., 2001, 2002; Williams et al., 2003).

Currently, various cell cultures have been used as a source of fresh tachyzoites in all aspects of research. In that sense, availability of different cell lines susceptible to the parasite would be of great interest for diagnostic and experimental usage of *N. caninum* and also useful for the production of its vaccine. Among these, Vero cell line are the most widely used in routine for the cultivation of the parasite tachyzoite *in vitro* (Vonlaufen et al., 2004; Lei et al., 2005; Kang et al., 2008).

However, the cultivation of these cells sometimes presents difficulties, especially with regard to the speed of propagation and the required level of nutritional components of the medium. Furthermore, one of the most limiting aspects in scaling up the parasite production on monolayer cell lines is that they are anchorage-dependent, and in absence of surface attachment interactions between the integrins and extracellular matrix, these cells do not proliferate (Werner et al., 1992; Assoian, 1997; Griffiths, 2001).

Recently, murine macrophage cell line was applied to study a wide range of intracellular infectious agents including *Mycobacte-*

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rium tuberculosis (Chan et al., 1992), *Brucella abortus* (Jiang et al., 1993), and *Cryptococcus neoformans* (Naslund et al., 1995).

In the present study the suitability of two cell lines including: J774 and Vero cell lines to support *in vitro* growth of *N. caninum* tachyzoites during continuous passages were compared. In addition, a preliminary study was performed in broiler chicken embryonated eggs to determine whether the type of host cell *in vitro* would lead to attenuation of virulence of *N. caninum* tachyzoites (live attenuated vaccine) *in vivo*.

2. Materials and methods

2.1. Cell lines and parasites

J774 and Vero cell lines were obtained from the Razi Vaccine and Serum Research Institute, Karaj, Iran. Before inoculation of parasites, host cells were incubated for 24 h. Both Vero and J774 cells were maintained in the same condition (at 37 °C in an atmosphere of 95% air and 5% CO_2). The tachyzoites of NC-1 isolate were cultured on a 24-h-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% CO_2 (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).

2.2. Comparison of yield of parasite derived from J774 and Vero cell lines

NC-1 tachyzoites (obtained from *in vitro* described above) were adjusted to a concentration of 2×10^6/ml in PBS and 1 ml was added to the cells in each 25 cm^2 tissue culture flask (Lv et al., 2010 with some modifications). Both cell lines were cultured at an equal concentration of 10^6 cells per each separate flask (parasite to cell ratio was 2 tachyzoites: 1 cell; three 25 cm^2 tissue culture flasks for each cell line). Cells infected with parasites were maintained in DMEM medium with 2% FBS, 2.30 mg/ml NaHCO3, 2.38 mg/ml HEPES, 2 mm glutamine, 50 U/ml penicillin, and 50 mg/ml streptomycin at 37 °C with 5% CO_2. Whole development process of *N. caninum* tachyzoites in each cell line (at the first inoculation) was observed daily under an inverted phase-contrast microscope (XDS-1B, China). The incubation was continued until the maximum number of tachyzoites was released from both host cells (for 4 consecutive days). The numbers of extracellular tachyzoites from 10 fields in each flask were counted. The daily average number of extracellular tachyzoites per flask was calculated (Lv et al., 2010), and growth curves of *N. caninum* tachyzoites *in vitro* were drawn.

2.3. Continuous N. caninum tachyzoites culture

Purified *N. caninum* tachyzoites grown *in vitro* were added to J774 and Vero cell lines. Before inoculation of parasites, host cells were incubated for 24 h. The cultures were examined daily by phase-contrast microscopy using an inverted microscope to determine the presence of released tachyzoites. When 80–90% of the host cells were disrupted and released a high concentration of tachyzoites, the flasks were sharply tapped from the sides to release the cells. The amount of tachyzoites harvested from each cell culture type in the 5 ml of decanted supernatant was concentrated by centrifugation at 500*g* for several minutes to eliminate the cell culture debris, and then again at 3000*g* for 10 min to concentrate the tachyzoites (Doskaya et al., 2006). Subsequently, tachyzoites harvested from two cultures supernatants were diluted with maintenance medium. Then, freshly grown J774 and Vero cells in 25 cm^2 flasks were inoculated with 10^6 tachyzoites in 1 ml of maintenance medium. Continuous production of *N. caninum* tachyzoites in two different cell culture types was performed for the same length of time (approximately 3 months). During this time, 23 passages were achieved for both cell cultures.

2.4. Virulence assay

Experimental infections were conducted in embryonated eggs from 70 Lohman broiler chickens randomly divided into seven equal groups. All eggs were maintained in an incubator with a controlled temperature, humidity and rotation until 9 days of incubation. The three groups included group V: tachyzoites- derived from Vero cell line, group J: tachyzoites- derived from J774 cell line and group C: control group. Then both groups of V and J were divided into three groups (V1, 2, 3 and J1, 2, 3) which were inoculated with different dilutions (0.5×10^4, 1.0×10^4, 1.5×10^4) of tachyzoites/embryonated egg. The 7th group was considered as the control and was inoculated with 150 µl sterile cell culture medium. Inoculation was performed in the chorioallantoic (CA) liquid of 9-day-old embryonated eggs (Mansourian et al., 2009). The embryos were observed twice daily until hatching and the number of deaths in each group was recorded daily.

2.5. Histopathological and immunohistochemical studies

Dead embryos were necropsied and suitable samples from different tissues including the liver, heart and brain were collected for histopathological studies. Live chickens after hatch were also observed and any clinical signs recorded. Euthanized chickens (on the first hatched day) were then necropsied and suitable samples from the mentioned tissues were collected and examined. For histopathological study the samples were fixed in 10% buffered formalin, embedded in paraffin, sectioned at about 5 mm, stained with hematoxylin and eosin and studied microscopically with a light microscope.

For IHC staining, the anti-*N. caninum* antibody (primary antibody) was produced by preparation of the killed tachyzoite *N. caninum* antigen (immunogen), estimation of the protein concentration, immunization of two rabbits subcutaneously with a mixture of antigen and freund's adjuvant in two injections with a two-week interval, and finally, detection of the polyclonal antibody titer by modified agglutination test (MAT). Consecutive sections to those used for histopathological examination were subjected to IHC. The IHC staining was performed based on the procedure of DakoCytomation Envision + Dual Link System- HRP (DAB+) kit (DakoCytomation, Denmark) with positive internal and external controls.

2.6. Molecular study

For molecular study small pieces (50 mg) of suitable samples from different tissues described above were removed and stored at −70 °C. DNA was extracted from the samples using the DNP™ Kit (Cinna-Gene) as per manufacturer's instructions. Then, following extraction the DNA was stored at −70 °C prior to analysis by PCR. Primer pair Np21/Np6 was used for amplification of the Nc-5 gene (Kang et al., 2009). The expected size of the amplicon with this primer pair is 328 bp. Each 25 µl PCR reaction contained 2.5 µl PCR buffer (10×), 3 mM MgCl2, 200 µM of dNTP, 400 nM of each primer and 4 µl of DNA. Thermal cycling was done as follows: 94 °C for 4 min (as initial denaturing), 25–30 cycles of 94 °C for

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