

## Original article

# Modifications in virulence of *Neospora caninum* after long term maintenance in murine macrophage

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## Summary

The aim of the present study was to attenuate *Neospora caninum* tachyzoites through continuous passage in murine macrophage and examine the virulence of attenuated tachyzoites in the mouse. The NC-1 reference isolate was 120 times passaged in the J774 cell line to produce attenuated strain. The virulence of tachyzoites was evaluated in a mouse model. Thirty-five female BALB/c mice randomly divided into seven groups. The first group, as control, was inoculated with culture media by subcutaneous injection. The second, third, and fourth groups received various dilutions ( $10 \times 10^6$ ,  $5 \times 10^6$ ,  $20 \times 10^6$ ) of acute NC-1 tachyzoites. Other groups were inoculated by attenuated tachyzoites with the same dilutions. The skin test and ELISA assay were performed to assess the cellular and humoral immunity responses. The mortality and clinical changes were noted daily. Notably, the significant difference was observed in the mortality rate of mice inoculated by acute and attenuated tachyzoites. Besides, serum antibody titers for *N.caninum* were higher in mice that received attenuated *N.caninum*. Also, mice that received live attenuated NC-1 showed significant footpad swelling. In conclusion, the present findings indicated that the culture of *N.caninum* in the J774 cells led to decrease of virulence of NC-1 significantly, which is confirmed here by the response of humoral and cellular immunity. This attenuated tachyzoites might be used as a candidate for further research to develop live vaccines against *N.caninum*.

**Keyword:** *Neospora caninum*, murine macrophage, serial passages, Balb/c mice

## Introduction

*Neospora caninum* was first recognized in 1984, which is an obligate, intracellular,

coccidian parasite of the phylum Apicomplexa (Lindsay et al., 1996). It has a worldwide distribution, which causes infection in several animals such as cattle, sheep, goat, deer, horse,

dog, cat, and mouse. Neonatal mortality and abortions are a significant obstacle in livestock operations, and neosporosis is a considerable cause of abortion in cattle. (Dubey and Lindsay, 1996). *N. caninum* is known as one of the most efficiently trans-placental transmitted parasites (Dubey et al., 2007). The parasite seriously impacts the economic performance of the dairy and beef industries (Reichel and Ellis, 2006). Currently, there is no suitable chemotherapeutic strategy to prevent trans-placental transmission. Also, to eradicate the parasite in cattle, producing of an effective vaccine highly desirable. It is believed that vaccination may be a conceivable strategy for the control of bovine neosporosis.

A previous study showed the effective attenuation of *N.caninum* via serial passages (during three months) in the J774 cell line by chicken embryonated eggs, which dramatically reduced the mortality rate in chicken embryonated eggs (Khordadmehr et al., 2013). In the present study, we evaluated the virulence of attenuated *N.caninum* in the mouse model (*in vivo*) by increase the passage time, which has been previously performed in different cell lines (*in vitro*) and chicken embryonated eggs (*in vivo*) (Khordadmehr et al., 2013). The embryonated chicken is partly appropriate for *N.caninum*, *Toxoplasma gondii*, and *Besnoitia* (Namavari et al., 2012; Setasimy and Namavari, 2015; Namazi et al.,

2010), and it is not convenient to produce immune responses. Since studies have shown the use of live attenuated strain as a vaccine has promising results (Monney and Hemphill, 2014), the present findings can be used for this purpose.

## Materials and methods

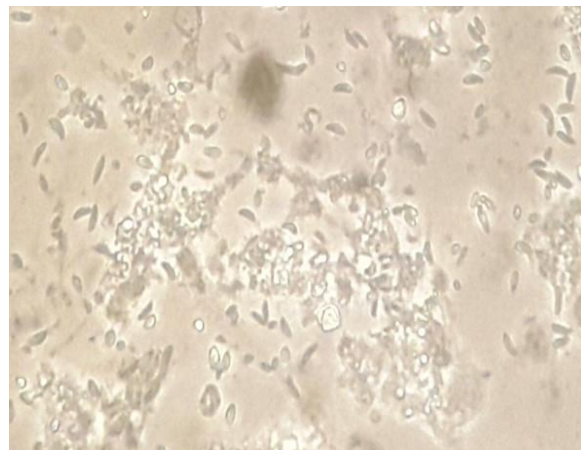
### *Cell line and parasite-derived from J774*

J774 cell line was obtained from Razi Vaccine and Serum Research Institute, which (Figure 1) was maintained in the condition of (37°C in an atmosphere of 95% air and 5% CO<sub>2</sub>). The tachyzoites of NC-1 isolate were cultured in J774 cell line and they maintained in DMEM Medium (Sigma Co., USA), supplemented with 2% fetal calf serum, penicillin/ml (10,000U), streptomycin/ml (100µg) and streptomycin (25µg) (Khordadmehr et al., 2013). The whole development process of *N. caninum* tachyzoites *in vitro* in J774 cells was studied daily using an inverted microscope. Tachyzoites were taken from infected cell cultures when approximately 80–90% of the J774 host cells had lysed (Figure 2), as indicated by microscopic observation of cytopathic effects (CPE), which subsequently counted using a hemacytometer (Chamberland and Current, 1990). The incubation was continued until the maximum number of

tachyzoites was released from host cells (Lv et al., 2010).



**Fig. 1.** J774 cell line  $\times 100$



**Fig. 2.** Released *N. caninum* tachyzoites from J774 cells  $\times 400$

### Mice and experimental design

Seven groups of five female Balb/c mice were inoculated subcutaneously. As more details, the first group, as control, received culture media, the second, third, and fourth groups received acute NC-1 isolate with  $5 \times 10^6$ ,

$10 \times 10^6$ ,  $20 \times 10^6$  tachyzoites in 500  $\mu$ l DMEM. The fifth, sixth, and seventh groups were inoculated with  $5 \times 10^6$ ,  $10 \times 10^6$ , and  $20 \times 10^6$  tachyzoites of attenuated NC-1 in 500  $\mu$ l DMEM. Mice were inspected daily for mortality and changes in clinical condition

or behavior (Kargar et al., 2011; Lunden et al., 2002).

### ELISA test

Three weeks after inoculation, the ELISA was used to establish the seroprevalence of anti-*N. caninum* antibody. ELISA was performed as previously described (Moore et al., 2005). Briefly, sera from each group were posited into 96-well plates coated with *N. caninum* tachyzoite antigen and diluted murine serum samples (1:50). Of note, all reactions were considered in duplicates. An ELISA reader was used (492 nm) for data analyses.

### Skin test

Footpad reaction was carried four weeks after inoculation according to Omata's method (Omata et al., 2006). Mice in each group were injected in the footpad with 50µl of PBS containing paraformaldehyde-fixed tachyzoites and the same volume of PBS injected to oppose the footpad. The average thickness of the hind footpad measured using a thickness gauge, 6 and 24 hours after injection (Omata et al., 2006).

### Molecular study

For the molecular study, tissue samples (50 mg) from the brain, liver, and heart tissues of mice (after euthanizing) were removed and stored at -70°C. DNA was extracted from all samples using the DNPT<sup>TM</sup> Kit (Cinna-Gene,

Iran) based on the manufacturer's instructions. Then, the extracted DNA was stored at -70°C for further analyses. Np21/Np6 primer pairs was used for amplification of the Nc-1 gene as follow (Kang et al., 2009; Okeoma et al., 2004):

Np21:5'-CCCAGTGCGTCCAATCCTGTAAC-3'

Np6:5'-CTCGCCAGTCAACCTACGTCTTCT-3'

The expected size of the amplicon with this primer pair is 328 bp (Kang et al., 2009; Okeoma et al., 2004). Each 25µl PCR reaction included (BioNeer, Korea):

- 2.5µl PCR buffer
- 3 mM MgCl<sub>2</sub>
- 200µM of dNTP
- 400 nM of each primer
- 4µl of DNA

Thermal cycling was conducted as follows:

- 94°C for 4 min (as initial denaturing)
- 25–30 cycles of 94°C for 50 s
- 53°C for 50 s
- 72°C for 1 min
- 5 min heating at 72°C (as the final extension)

Finally, six µl of PCR product was electrophoresed on 2% agarose gel.

### Statistical analyses

For statistical analysis of the present data, GraphPad Prism software (GraphPad Prism

4.0, San Diego, CA) was used. The statistically significant differences between the experimental groups were evaluated by Student's t-test (ELISA data) and Fisher exact test (for mortality rate and PCR data), and a  $p < 0.05$  was considered statistically significant.

## Results

### Parasite attenuation

The present results presented rapid attenuation of virulence of *N. caninum* tachyzoites by continuous passages of parasites in J774 suspension cell line after 120 times during six months. These results suggest that it is possible to attenuate the virulence of tachyzoites through prolonged *in vitro* cultivation in J774 cell line.

### Mortality rate

All mice of the control group and groups that received attenuated NC-1 remained alive. Mice that received  $10 \times 10^6$  and  $20 \times 10^6$  acute NC-1 had been died up to the second week after inoculate. Only the group that inoculated with  $5 \times 10^6$  survived but showed infection symptoms, such as reluctance to move and displayed rough hair coat (Figure 3). On the other hand, all mice in groups inoculated with  $5 \times 10^6$ ,  $10 \times 10^6$ , and  $20 \times 10^6$  tachyzoites of attenuated NC-1 stayed alive until the end of the experiment, and there were no symptoms of infection in them (Table 1). This significant difference in mortality rates clearly demonstrated the effect of the J774 cell line on the attenuation of virulence of *N. caninum* tachyzoites.



**Fig. 3.** Balb/c mouse infected experimentally by *N. caninum* which showed the infection symptoms such rough hair coat

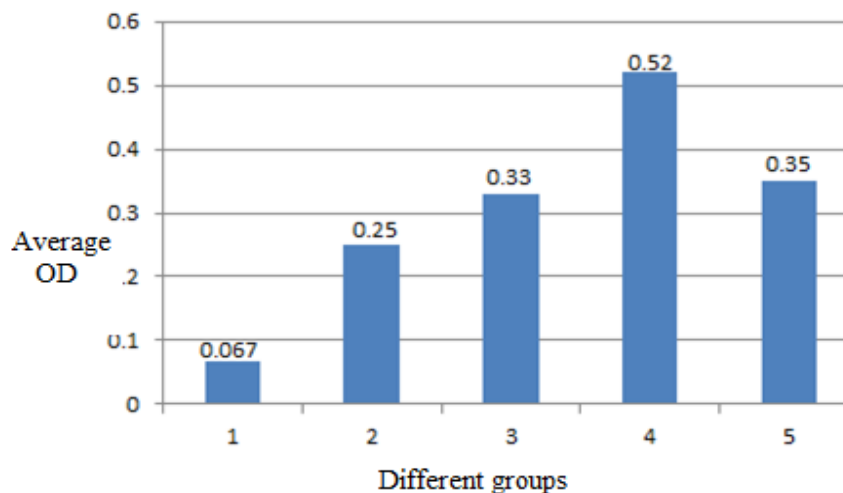
**Table 1.** Mortality results in different experimental groups (n = 5).

Groups	Dose of inoculation	10 days after inoculation	14 days after inoculation	18 days after inoculation	Total
Acute tachyzoite	5×10 <sup>6</sup>	0	0	1	1
	10×10 <sup>6</sup>	0	2	3	5
	20×10 <sup>6</sup>	5	0	0	5
Attenuated tachyzoite	5×10 <sup>6</sup>	0	0	0	0
	10×10 <sup>6</sup>	0	0	0	0
	20×10 <sup>6</sup>	0	0	0	0
Control	DMEM	0	0	0	0

### ELISA findings

Serum antibody titers for *N.caninum* were higher in mice that received attenuated *N.caninum* than control mice or mice that received acute NC-1. Based on the *t*-test

examination, a significant difference ( $P < 0.0001$ ) was found between high passage tachyzoites and acute tachyzoites groups (figure 4).

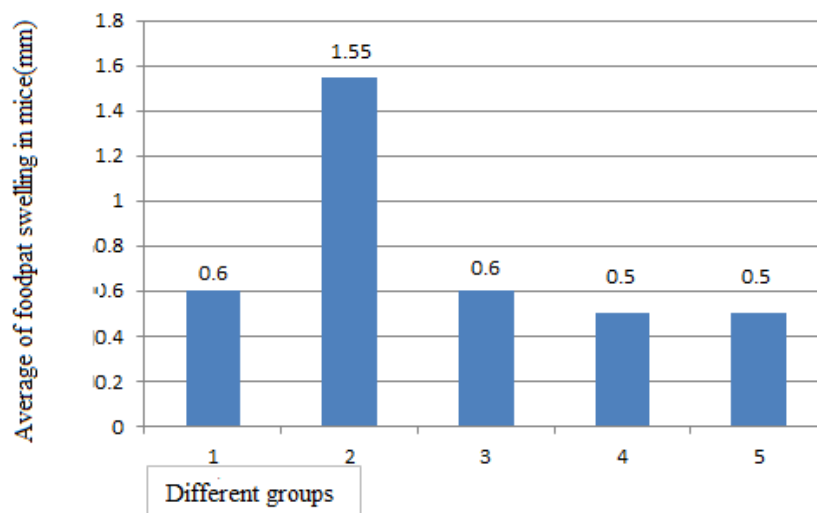


**Fig. 4.** Results of the ELISA test. 1) Control group, 2) 5×10<sup>6</sup> (Acute tachyzoite), 3) 5×10<sup>6</sup> (Attenuated tachyzoite), 4) 10×10<sup>6</sup> (Acute tachyzoite), 5) 20×10<sup>6</sup> (Attenuated tachyzoite). The minimum and maximum of OD were observed in the control and 10×10<sup>6</sup> Attenuated tachyzoite groups, respectively.

### Skin test

Mice that received live attenuated NC-1 showed significant footpad swelling, while there was no significant swelling in the mice

that received acute NC-1. In all mice, the mean increase in the thickness of the swelling peaked at 24h, about 1.2-fold of the thickness after injection (Figure 5).

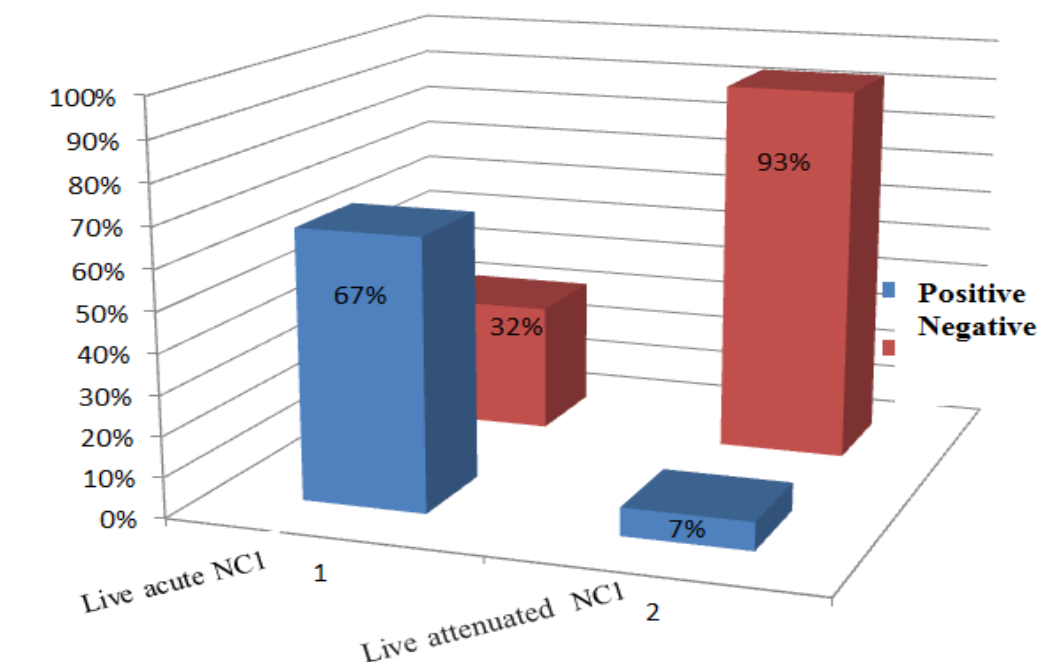


**Fig. 5.** The results of the footpad thickness 24h after skin test. 1)  $5 \times 10^6$  (Attenuated tachyzoite), 2)  $10 \times 10^6$  (Attenuated tachyzoite), 3)  $20 \times 10^6$  (Attenuated tachyzoite), 4)  $5 \times 10^6$  (Acute tachyzoite), 5) Control group. (Mice in other groups have died before skin test). The thickness of swelling in group received  $10 \times 10^6$  Attenuated tachyzoites is significantly higher than other groups.

### Molecular finding

The results of the molecular study demonstrated the presence of parasites in the heart, liver, and brain of mice that received acute NC-1. A significant reduction of the presence of parasite DNA in the brain, liver, and heart samples were detected in all groups that received live attenuated NC-1 in

comparison with other groups. 93% of mice inoculated with attenuated tachyzoites showed negative results in PCR assay, while in groups inoculated with acute tachyzoites, only 32% were considered as negative for the presence of the parasite genome (figure 6). There was a significant difference between acute and attenuated tachyzoites groups ( $P < 0.05$ ).



**Fig. 6.** The results of the PCR test in acute and attenuated tachyzoites groups. A significant difference was observed between two groups ( $P < 0.05$ ).

## Discussion

This study represents the J774 cell culture as a suspension cell line that has been used successfully for cultivating *N.caninum* tachyzoites. To date, numerous host cells, such as Vero cell, bovine endothelial cell bovine angio-endothelial cell, cat and dog fibroblast cell, cat kidney cell, MCF-7 have been fortunately applied for the laboratory maintenance, propagation, and passage of *N.caninum* tachyzoites (Okeoma et al., 2004; Omata et al., 2006; Lei et al., 2005; Lv et al., 2010). In this regard, the Vero cell line, as an adherent cell line, is the most frequently used

for *in vitro* experiments (Dubey, 2003). However, the cultivation of adherent cells has some difficulties, particularly concerning the speed of propagation. Thus, the availability of suspension culture susceptible to *N.caninum* would be of great interest in the mass production of *N. caninum* tachyzoites. It was previously reported that the J774 cell line is introduced as a suitable host cell for cultivating *N. caninum* (Khordadmehr et al., 2013). Considering the key role of macrophages in the defense mechanisms of the immune system, it is not surprising that continuous cultivation of



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*N. caninum* tachyzoites led to the attenuation of virulence of this protozoan.

Here, serum antibody titers of *N. caninum* were higher in mice that received live attenuated *N. caninum* than in control mice. Increases in footpad thickness in mice that received live attenuated *N. caninum* suggested cellular immunity. This finding was similar to the previous findings reported by Omata et al. and Nishikawa et al., who described that mice infected with *N. caninum* produce a delayed-type hypersensitivity reaction (DTH), which is a good indicator of the development of type 1 immune responses (Omata et al., 2006; Nishikawa et al. 2003).

Some live protozoan vaccines (like *Leishmania major*, *Leishmania Mexicana*, *Babesia bovis*, and *Babesia bigemina*) were successfully produced by attenuating parasite virulence (Daneshvar et al., 2003; Pipano et al., 2002). In this connection, *N. caninum* is similar in biological characteristics to *T. gondii*, and recent vaccine against *T. gondii* has been carried out using an attenuated (S48) strain of *T. gondii* (Wilkins et al., 1988). This vaccine is developed by serial passage of the S48 strain of *T. gondii* over 3000 times in mice (Buxton, 1993). The successful experience of the Toxoplasma vaccine as a commercial vaccine to prevent ewe abortions demonstrates the development of a live attenuated vaccine that can be administered for *N. caninum*.

Thus, the results of the present and a previous study (Khordadmehr et al., 2013) can be used to produce a live attenuated vaccine for *N. caninum*. It has been previously indicated that prolonged *in vitro* cultivation of *N. caninum* tachyzoites resulted in a reduction in virulence *in vivo* (Long et al., 1998). Notably, a commercial inactivated vaccine called NeoGuard™ was only moderately successful in field trials (Weston et al., 2012). An efficacious and safe vaccine that can limit congenital infection of cattle with *N. caninum* is not currently available. Based on the appropriate protection rates achieved by live attenuated *N. caninum* we hope this study provides the base for further research to produce an effective vaccine for *Neospora caninum*.

### Conclusion

In conclusion, the present findings indicated that the culture of *Neospora caninum* in the J774 cell line led to a decrease of virulence of NC-1 significantly, which is confirmed here by the response of humoral and cellular immunity. The most considerable aspect of the present results is confirming the previous findings, which have been evaluated in the chicken embryonated eggs. Indeed, here, we approved the attenuation of the long term passages of NC-1 in a mouse model, which can be used as a suitable *in vitro* model for mass

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production of parasite and attenuation of virulence of *N. caninum* for live vaccine development.

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### Conflict of interest statement

There is no conflict of interest.

### Ethical approval

All animal works were performed in accordance with the National Institutes of Health (NIH) Guidelines for the use of experimental animals, and the study protocol was approved by the Animal Ethic Committee at Razi Vaccine and Serum Research Institute, Tehran, Iran.

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