*Full Article*

# Virulence assessment of a *Neospora caninum* isolate for inbred C57BL/6 mouse

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**ABSTRACT**

*Neospora caninum* (*N. caninum*) is an apicomplexan parasite and causes abortion and congenital neosporosis in cattle worldwide. In this study, we evaluate the virulence of a *N. caninum* isolate on mouse strain C57BL/6. Six groups of five mice C57BL/6 were intraperitoneally inoculated with $1 \times 10^7$, $1.5 \times 10^7$, $2 \times 10^7$, $3 \times 10^7$ and $4 \times 10^7$ tachyzoites and a control inoculum of DMEM, respectively. Clinical signs and mortality rate were recorded and confirmed by histopathological findings and molecular method. The results of this study indicated that LD50 was $2.5 \times 10^7$ tachyzoites of *N. caninum* per C57BL/6 mouse. This can be used as a lethal challenge model in vaccine development studies.

*Keywords:* *Neospora caninum*, C57BL/6 mouse model, LD50

## INTRODUCTION

*Neospora caninum* (*N. caninum*) is a cyst-forming coccidian parasite closely related to *Toxoplasma gondii* species. It has been shown to be a serious cause of abortion in cattle (Dubey & Schares 2006). Considerable economic losses are attributed to *N. caninum* in the farming industry, including the costs of still birth and neonatal mortality, born clinically normal but with chronic infection, increased calving interval resulting from early fetal death and reduced milk production (Dubey *et al* 1996, Trees *et al* 1999, Dubey 2003, Botelho *et al* 2007). The dairy and beef industries in several parts of the world are severely affected by this disease (Dubey 1999, Wouda 2000, Ramamoorthy

*et al* 2007). Reproductive losses and abortion due to *N. caninum* infection has been reported in cattles in Iran (Habibi *et al* 2005, Malmasi *et al* 2007, Razmi *et al* 2006 and 2007). Mice have been commonly used as a model in experiments conducted to study the biology of *N. caninum* and develop vaccine against this infection (Lindsay *et al* 1990, 1999, Cannas *et al* 2003, Bartley *et al* 2006). The C57BL/6 mice are very well suited for *N. caninum* studies (Long *et al* 1998, Botelho *et al* 2007, Ramamoorthy *et al* 2007).

LD50 is one of the pathogen characters and a widely accepted tool in vaccine and toxicity studies. It is of particular use to manufacturers as it is prescribed in most international pharmacopeias as a test to measure the efficacy of vaccines and drugs (Rabouhans 1986, Botham 2002, Ramamoorthy *et al* 2005). On other hand pervious study demonstrated that the number of

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passages of *N. caninum* in vitro could affects its virulence (Bartley *et al* 2006). The present study determined the LD50 of *N. caninum* strain NC-1 isolated at Razi vaccine and serum research institute, for the mouse strain C57BL/6.

## MATERIALS AND METHODS

**Parasite culture.** Vero cells were cultured in DMEM Medium (Sigma Co., USA), containing 2% fetal calf serum, penicillin/ml (10,000 U), streptomycin/ml (100 µg) and amphotericin (25 µg, Invitrogen; USA) with 5% $CO_2$ in tissue culture, kept at 37 °C in flasks and were trypsinized 1 to 3 times a week. *N. caninum* (strain NC-1) tachyzoites were cultured on the monolayer spread of the Vero cells (Dubey *et al* 1988, Dubey 1999, Dubey & Schares 2006). Intracellular parasites were harvested by trypsinization of infected Vero cells about 4-5 days post infection, after lysis of the Vero cells (60-80%). Free tachyzoites were removed from the tissue culture flasks by collecting the medium supernatant. For preparation of the necessary inoculum doses ($1 \times 10^7$, $1.5 \times 10^7$, $2 \times 10^7$, $3 \times 10^7$ and $4 \times 10^7$), parasites were pelleted by centrifugation (2,000 g for 10 min) concentration set with a Neubauer chamber and diluted in DMEM medium.

**Infection of Mice.** Thirty female C57BL/6 mice were divided into 6 groups of five 6–8 week old mice, that each was inoculated intraperitoneally (IP) with $1 \times 10^7$, $1.5 \times 10^7$, $2 \times 10^7$, $3 \times 10^7$ and $4 \times 10^7$ tachyzoites and a control inoculum of DMEM, respectively (Table 1). Mice were observed and monitored daily for 28 days for clinical signs and mortality.

**Histopathologic and PCR examination.** At necroscopy, 20 mg of the brain tissue was weighed into sterile microtubes and stored frozen at -20 °C until for PCR assay. The rest of the brain material was fixed in 10% buffered formalin and procssed for routine paraffin embedding and haematoxylin and eosin (H&E) staining of 5µm thin sections. The used protocol for DNA extraction and PCR assay of brains samples was

as the method previously described by Yamage et al (1996).

**Statistical analysis.** The LD50 was calculated by the Reed and Muench method (Pizzi 1950).

**Table 1.** Experimental design.

| Group | Number of mice | Intraperitoneally (IP) inoculum NC tachyzoite |
|---|---|---|
| 1 | 5 | $1 \times 10^7$ |
| 2 | 5 | $1.5 \times 10^7$ |
| 3 | 5 | $2 \times 10^7$ |
| 4 | 5 | $3 \times 10^7$ |
| 5 | 5 | $4 \times 10^7$ |
| 6 | 5 | PBS |

**Table 2.** LD50 titration of N. caninum tachyzoites

| Mouse number | Dose of tachyzoites | | | | |
|---|---|---|---|---|---|
| | $1 \times 10^7$ | $1.5 \times 10^7$ | $2 \times 10^7$ | $3 \times 10^7$ | $4 \times 10^7$ |
| 1 | K28 | K28 | K28 | D6 | D3 |
| 2 | K28 | K28 | K28 | D6 | D3 |
| 3 | K28 | K28 | K28 | D7 | D4 |
| 4 | K28 | K28 | K28 | D9 | D5 |
| 5 | K28 | K28 | K28 | D9 | D5 |

D: Died; K: killed; number: number of days to death or euthanasia

## RESULTS

All the mice in the $2 \times 10^7$ group survived and none of the mice in the $3 \times 10^7$ group survived. Data from LD50 titration is represented in Table 2. The LD50 as determined by the Reed and Muench method was $2.5 \times 10^7$ tachyzoites per mouse. In our study, all the mice administered $3 \times 10^7$ and $4 \times 10^7$ tachyzoites died between days 3-9 PI., while mice administered $1 \times 10^7$, $1.5 \times 10^7$ and $2 \times 10^7$ were survived. Most of the mice from groups 2 and 3 showed clinical sings of disease that they were reluctant to move and displayed rough hair coats. Also, at day 9, two mice from group 3 showed other clinical symptoms including hunching and pelvic limb weakness. Further, no clinical signs have been observed in mice infected with $1 \times 10^7$ tachyzoites. The control group showed no clinical symptoms or mortality.

The most significant lesions found on histopathological examination of brain tissues revealed mild to moderate lymphoplasmacellular meningoencephalitis, lymphoplasmacellular perivascular cuffing (PVC) (Figure 1) and focal glial cell activation. In the PCR assay brain samples from these infected mice produced a single product of 328 bp similar to those of the positive control while negative control was PCR negative (Figure 2). Histopathological examination of brain samples from mice that died from $3 \times 10^7$ and $4 \times 10^7$ tachyzoites inoculom did not show any pathological changes in the brain**.**
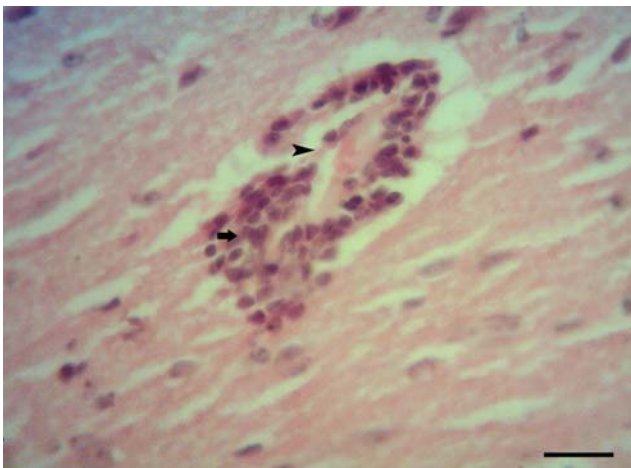


**Figure1.** Histopathological lesions of brain in a mouse experimentally infected with $2 \times 10^7$ tachyzoites Histopathological lesions of brain in a mouse experimentally infected with $2 \times 10^7$ tachyzoites. A: gliosis (arrow), B: perivascular cuffing (PVC, arrow) by monoculear cells (H and E, scale bar: 85 μm).

## DISCUSSION

Determining the LD50 value eliminates the need for a long waiting period for the analysis of tissue samples by histological or PCR methods (Rabouhans 1986, Botham 2002, Ramamoorthy *et al* 2005). In this study LD50 was determined to be $2.5 \times 10^7$ tachyzoites per C57BL/6 mouse that shows lower virulence compared with other study which the LD50 was calculated as $1.5 \times 10^7$ tachyzoites of NC1 per mouse (Ramamoorthy *et al* 2007). The NC-1 is the most common laboratory strain of *N. caninum*, so this difference between the LD50 are not entirely surprising since the passage number, condition of culture and even the

concentration of antibiotics affects on the virulence of tachyzoite and the possibility of difference in resistance of C57BL/6 mice (Daneshvar *et al* 2003, Bartley *et al* 2006). In this study, the most common clinical sings of disease in mice were related to central nervous system and include reluctant to move, hunching and pelvic limb weakness. On other hand, the main histopathological changes, found on histopathological examination of brain tissues of the animals of 3 group, were focal glial cell activation, lymphoplasmacellular meningoencephalitis and perivascular cuffing (PVC). Similar histological observations to those described in our study were seen by Long et al (1998), Lindsay et al (1995, 1999), Lunde´n et al (2002), Bartley et al (2006) and Ramamoorthy et al (2007), when examining CNS tissue from infected mice.
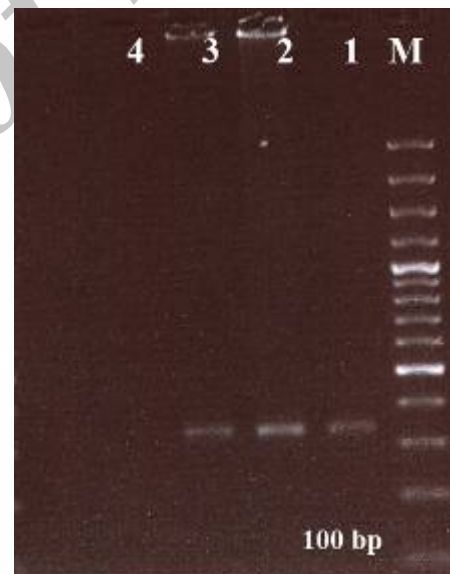


**Figure 2.** Detection of N. caninum-specific DNA in infected mice brains. Agarose gel electrophoresis of N. caninum-specific PCR products, amplified from DNA samples collected from brains of group 3 infected mice with $2 \times 10^7$ tachyzoites (Lane 2 and 3). Negative (Lane 4; brain from uninfected mice) and positive (Lane 1) control samples and DNA ladder standards (M) were also shown.

Histopathological evaluation of brain tissues from the animals of 3 and 4 group did not show any pathological changes, possibly because the time from inoculation to death was short and death occurred shortly, before brain lesions could develop. Similar findings have been

reported by using PCR to quantify infection intensitie in brain tissue (Eperon *et al* 1999, Gottstein *et al* 2001).

To our knowledge, C57BL/6 mice exhibit clinical signs and mortality due to Neosporosis and are, therefore, an invaluable tool in for determine the LD50 of *N. caninum* in mice.

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