Oral infection of calves with *Neospora caninum* oocysts from dogs: humoral and cellular immune responses

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Received 7 June 1999; received in revised form 26 August 1999; accepted 7 September 1999

**Abstract**

*Neospora caninum* has been identified as a major cause of abortion in cattle in a number of countries throughout the world. Until the recent demonstration that dogs can serve as a definitive host of this parasite, it was not possible to study the infection in cattle orally exposed to oocysts. The aim of this study was to investigate the potential of *N. caninum* oocysts to infect calves, and to define initial immune responses that arise after oral infection. Seven calves were fed approximately \(10^4-10^5\) *N. caninum* oocysts, three calves served as uninfected controls. Before infection, all calves were serologically negative for anti-*Neospora* antibodies and the calves were non-reactive to *Neospora* antigen in an in vitro lymphocyte proliferation assay. Peripheral blood lymphocytes from inoculated calves were able to mount in vitro proliferative responses to crude *N. caninum* antigen extract as early as 1 week p.i. Within 2 and 4 weeks p.i., *Neospora*-specific IgG1 and IgG2 antibodies were detected by IFAT and ELISA in serum from infected calves but not from sham-infected calves. The continued presence of reactive cells in the blood, spleen and mesenteric, inguinal, bronchial lymph nodes was seen as late as 2.5 months p.i., and parasite DNA was detected in the brain and spinal cord of the infected animals by PCR, indicating that the cattle were infected by oral inoculation of *N. caninum* oocysts collected from dogs, and that the animals were systematically sensitised by parasite antigen. 1999 Published by Elsevier Science Ltd.

**Keywords:** Agglutination test; Antibodies; Cattle; ELISA; IFAT; Immune response; *Neospora caninum*; Oocyst; Oral; PCR

**1. Introduction**

*Neospora caninum* is a tissue cyst-forming coccidian parasite that can infect and cause disease in a variety of mammalian genera [1]. In addition, *N. caninum* is recognised as a major cause of infectious bovine abortion in many parts of the world [2–7].

*Neospora caninum* is closely related to *Toxoplasma gondii*, which it resembles and with which it had earlier been confused [8]. Until recently, only the tachyzoites and tissue cysts of *N. caninum* had been described. Tachyzoites
develop intracellularly in many cell types, including neural and dermal cells. Tissue cysts have been found only in neural tissues, and are infectious when given orally. Recently, the Neospora life-cycle has been completed in the dog, which sheds oocysts upon feeding with tissue cysts [9, 10].

In cattle, it appears that one major route of transmission of N. caninum is transplacental, resulting in the birth of congenitally infected progeny [1]. Some investigations of neosporosis outbreaks in cattle herds suggest an external source of infection, although neither oral infection with oocysts from the environment nor infection via contact with infected tissue has been demonstrated [11, 12].

The aim of the present study was to investigate the potential of N. caninum oocysts to infect calves when given orally, and to investigate the antibody and cellular responses of these calves. To our knowledge, this is the first report of an oral experimental infection of calves with N. caninum oocysts, as until now, cattle have only been experimentally infected by parenteral inoculation with N. caninum tachyzoites or tissue cysts [13–17].

2. Materials and methods

2.1. Animals

Ten Holstein steers (2.5 months old) were used in this study. Seven (numbers 334, 335, 338, 342, 344, 345, 346) were orally inoculated with approximately 10⁴–10⁵ N. caninum oocysts, and three calves (337, 339, 343) were sham-inoculated with water only. Blood was collected from the jugular vein weekly for leukocyte isolation and serum collection. For preparing the serum, blood was centrifuged at 1000 g for 10 min, and the serum was collected and stored at −20°C until use. At 76 days and 82 days p.i., the calves were killed and the following tissues were collected for lymphocyte isolation: mesenteric lymph nodes, bronchial lymph nodes, inguinal lymph nodes and spleen. The following samples were taken for immunohistochemical staining and PCR: brain (cerebrum, cerebellum, pons, medulla), spinal cord, heart, liver, lung, diaphragm, spleen, mesenteric lymph nodes, adrenals, kidneys, skeletal muscles, rumen, reticulum, omasum, abomasum and small intestine.

2.2. Parasites and antigen preparation

Neospora caninum oocysts were obtained from faeces of an adult female mix-breed dog. The bitch had no demonstrable antibodies to N. caninum in 1:25 dilution of serum tested by IFA. The dog was fed brains of 10 outbred cortisone-treated mice [18] injected subcutaneously with approximately 10⁴ tachyzoites of the NC-2 isolate [19] 19 days previously. The dog shed oocysts 7–10 days p.i., but only in low numbers. Oocysts were seen in sugar faecal floats using 10 g of faeces, but were too few to count. The remaining faeces were suspended in 2% H₂SO₄ and, after straining through a 90 μm metallic sieve, the filtrate was aerated at 20–22°C for 1 week. The infectivity of the inoculum was verified by feeding 1/10 of the faecal suspension given to each calf to four Swiss–Webster female mice (25 g). The mice had been given 10 μg ml⁻¹ dexamethasone in their drinking water beginning 9 days before until 7 days p.i. of oocysts. In addition, the suspension of oocysts was bioassayed in two gamma-interferon knockout (γ-IFN KO) mice as described [9]. Sporulated oocysts were seen in the inoculum fed to calves, but because of their low number and the high amount of faecal debris, it was not possible to count them in a haemocytometer, the sensitivity of which is 10⁴ oocysts ml⁻¹. Thus, the number of oocysts in the inoculum given to each calf was considered to be between 10⁴ and 10⁵.

Tachyzoites of the NC-2 isolate were disrupted using a Polytron (Kinematica) (6 min, speed 6) on ice, centrifuged at 20,000 g for 30 min and dialysed overnight against PBS (pH 7) using a membrane with a 10 kDa cut off (Spectrapor). Extracts were sterilised by filtration (0.22 μm, Costar) and were kept at −20°C until use. The protein concentration was determined using the Bio-Rad protein assay.
2.3. Immunohistochemical staining

The samples were fixed in 10% buffered neutral formalin. Paraffin sections were cut at 5 μm thickness and examined after staining with H&E. Deparaffinised sections were reacted with anti-*N. caninum* antibodies using reagents and methods as described [20].

2.4. Polymerase chain reaction detection of *N. caninum*

To reduce the potential of false-positive signals due to contamination of tissue samples at the time of the necropsy, sections of brain and spinal cord for PCR analysis were cut from the middle of tissue blocks. DNA was extracted from tissue samples (set 1 from 0.5 g tissue; set 2 from 2 g tissue) using a previously described technique [21].

Parasite DNA in bovine tissues was detected by PCR amplification of a 337 nt long fragment of Nc5 *N. caninum*-specific genomic DNA [22] using primers Np21plus and Np6plus [23]. The PCR was performed and products visualised on ethidium bromide stained polyacrylamide gels essentially as described [21], except that the number of amplification cycles was increased to 40 for a higher level of sensitivity. DNA (250 ng) from each bovine sample was used in each PCR. A minimum of two reactions for each sample for each set of extractions were performed on separate occasions. To avoid false-positive reactions, the phases of DNA extraction, PCR sample preparation and mixing of PCR reagents with DNA samples were performed in separate locations. Aerosol barrier tips, single-use plastic wear and disposable gloves were used. Distilled water was used as a negative control in every set of PCRs performed. NC-1 and NC-2 DNA extracted from tachyzoites were used as positive controls. To ensure that amplification of *N. caninum* DNA was not inhibited by factors co-puriﬁed during the DNA extraction procedure, resulting in a false-negative assignment, the PCRs were ‘spiked’ with a competitor molecule, pNc5C+ (Liddell et al., this volume).

2.5. Indirect immunofluorescence antibody test and the *N. caninum* agglutination test (NAT)

Sera were examined by IFAT as described [15, 24] and by NAT as described [25]. The NC1-isolate was used for IFAT and NAT. Sera were diluted two-fold starting at 1:25 for IFAT and 1:40 for NAT.

2.6. Whole tachyzoite lysate ELISA

*Neospora caninum* tachyzoite extract was diluted to 10 μg ml⁻¹ in sodium carbonate buffer (pH 9.6) and 100 μl (1 μg/well) was added to wells of 96 microtitre well plates. The plates were incubated overnight at 4°C, washed and then blocked with 5% horse serum in PBS containing 0.05% Tween-20 (PBST) for 1 h at 37°C. After washing, 100 μl of diluted sera (1/160) was added in duplicate wells, and the plates were incubated for 1 h at 37°C. Following three washes with PBST, 100 μl of polyclonal rabbit anti-bovine IgG1, IgG2, IgM or IgA (generous gift from Dr Guidry, USDA, Beltsville, MD) (1/1000) was added, and the plates were incubated for 1 h at 37°C. After washing (three times with PBST), 100 μl of goat anti-rabbit IgG conjugated to alkaline phosphatase (Kirkegaard & Perry) was added in the wells, and the plates were incubated for 1 h at 37°C. Following three washes with PBST, 100 μl of phosphatase substrate (1 mg ml⁻¹, Sigma) was added to the wells, and plates were incubated for 15 or 30 min for IgG1, IgM and IgG2, IgA detection, respectively. Optical density was measured at 405 nm.

2.7. Recombinant antigen ELISA

The recombinant antigen ELISA [26] was performed as modified [27]. Pooled recombinant NCDG1 and NCDG2 antigens were used to coat ELISA plates.

2.8. Leukocyte isolation and culture

Peripheral blood leukocytes were isolated over a density gradient using Ficoll-Hypaque. Gradients were centrifuged at 4°C for 45 min at
1000 g. After three washes with HBSS (pH 7) without calcium and magnesium, the cells were resuspended in RPMI supplemented with 5\times10^{-5} M 2-mercaptoethanol, 25 mM Hepes, 100 U ml^{-1} penicillin, 100 \mu g ml^{-1} streptomycin, 50 \mu g ml^{-1} kanamycin and 5\% FCS (5\% RPMI) at a concentration of 4\times10^6 cells ml^{-1}. The spleen and the mesenteric, bronchial and inguinal lymph nodes were minced with blunt scissors and forceps in HBSS on ice. Debris and clumps of cells were removed by gravity sedimentation for 10 min. The resulting cell suspensions were washed and resuspended in 5\% RPMI at a concentration of 4\times10^6 cells ml^{-1}. Aliquots of the peripheral blood, lymph node and spleen lymphocytes were stained for flow cytometry analysis.

To evaluate the proliferative capacity of the isolated cells, 4\times10^5 cells/microwell were stimulated with the mitogen ConA at a final concentration of 2.5 \mu g ml^{-1}. *Neospora caninum* tachyzoite extract was added at final concentrations of 10 or 2 \mu g ml^{-1}. Each stimulation was performed in triplicate. Cells were incubated for 4 or 5 days at 38^\circ C in a humidified atmosphere containing 5\% CO_2 in air. Proliferation was measured by incorporation of tritiated thymidine (0.5 \mu Ci for 6–8 h) and expressed as c.p.m. The *N. caninum* or ConA-specific proliferation was calculated by subtracting the mean medium-induced c.p.m. (=background) from the mean *Neospora* or ConA-induced c.p.m., respectively.

2.9. Flow cytometry

 Immunofluorescence staining of leukocytes was performed as described [28]. Monoclonal antibodies (VMRD) and their specificities are described in Table 1. The mAb PT85 which recognises a monomorphic determinant on class I antigens was used as a positive control, while background staining levels were determined with control mouse ascites fluid. After staining, cells were analysed with a flow cytometer Epics profile II (Coulter). Cells expressing the desired determinates are presented as the percentage of the total number of analysed cells.

2.10. Statistical analysis

Analyses of the whole tachyzoite lysate ELISA utilised a mixed linear model with fixed effects for treatment, date, and treatment by date interaction, and random effect attributable to animals. Independent two-sample $t$-tests were carried out to compare the percentage of cell subpopulations between infected animals and uninfected control animals. A probability value of less then 0.05 was taken to indicate that the values of the two treatments were not similar.

3. Results

3.1. Infection with *N. caninum* oocysts

The material fed to calves was lethal to both \gamma-IFN KO mice and at least one of four outbred mice. The \gamma-IFN KO mice died of neosporosis 11 and 12 days p.i. Two of the four outbred mice died on days 5 and 7 due to bacterial infection. One mouse was killed when ill 19 days p.i., and had *N. caninum* tachyzoites in brain and liver. One mouse was not infected.

3.2. Detection of *N. caninum* by immunohistochemical staining and PCR

Lesions consistent with neosporosis were not seen in tissues from any calves. *Neospora* parasites were neither seen nor demonstrable by immunohistochemical staining.

Table 2 shows the results of PCR assays performed on *N. caninum* DNA extracted from a portion of brain and spinal cord from each calf. *Neospora caninum* DNA was detected in all of the infected animals, whilst the uninfected animals gave negative results. Positive signals were apparent only after the number of amplification samples was increased to 40. *Neospora caninum* DNA was not always detected in every repeat reaction with the same sample. Polymerase chain reaction analysis performed on two different portions of the same tissue did not always give the same result (e.g. cow 344 brain was strongly positive in set 1, but in set 2 it was negative).
3.3. Indirect immunofluorescence antibody test and NAT

All calves were seronegative to *N. caninum* antibodies before feeding oocysts. The three control calves remained seronegative throughout the study. All calves fed oocysts developed antibodies to *N. caninum*, detected by NAT as well as by IFAT (Table 3), except one of the inoculated animals (345) in which antibodies to *N. caninum* were detected only by NAT, not by IFAT.

3.4. Antibodies against *N. caninum* tachyzoites

In the calves infected with *N. caninum* oocysts, anti-*N. caninum* IgG1 was first detected 2 weeks p.i. (Fig. 1a, *P* < 0.099) and anti-*N. caninum* IgG2 4 weeks p.i. (Fig. 1b, *P* < 0.097). Anti-*N. caninum* IgG1 and IgG2 levels were significantly different (*P* < 0.05) in infected calves compared with uninfected control calves by weeks 4 and 5, respectively. Anti-*N. caninum* IgM was detected 2 weeks p.i. (Fig. 1c, *P* < 0.0006), but decreased by 1 month p.i. to pre-infection levels (Fig. 1c, *P* < 0.821). No differences in IgA level were detected between infected animals and uninfected control animals (results not shown, *P* always >0.386). One (calf 345) of the seven infected animals did not show detectable anti-*N. caninum* IgG1, IgG2, IgM or IgA responses. The uninfected control calves did not show detectable anti-*N. caninum* IgG1, IgG2, IgM or IgA responses.

<table>
<thead>
<tr>
<th>Table 1</th>
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<td>Characteristics of the monoclonal antibodies to bovine mononuclear cells</td>
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<tr>
<td>Bovine antigen</td>
</tr>
<tr>
<td>----------------</td>
</tr>
<tr>
<td>MM1A</td>
</tr>
<tr>
<td>CACT138A</td>
</tr>
<tr>
<td>CACT80C</td>
</tr>
<tr>
<td>CACT116A</td>
</tr>
<tr>
<td>CACT61A</td>
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<tr>
<td>BAQ155A</td>
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<tr>
<td>CAM36A</td>
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</tbody>
</table>

3.5. Antibodies against recombinant antigens

In calves infected with *N. caninum* oocysts, positive IgG titres to recombinant tachyzoite antigens were detected as early as 2 weeks p.i. (Fig. 2). However, one (calf 345) of the seven inoculated animals did not show a detectable

<table>
<thead>
<tr>
<th>Table 2</th>
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<tr>
<td>Detection of <em>Neospora caninum</em> DNA in bovine tissues by PCR amplification</td>
</tr>
<tr>
<td>Calf number</td>
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<tr>
<td></td>
</tr>
<tr>
<td>Set 1</td>
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<tr>
<td>--------</td>
</tr>
<tr>
<td>334</td>
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<tr>
<td>335</td>
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<td>338</td>
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<td>342</td>
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<td>344</td>
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<td>345</td>
</tr>
<tr>
<td>346</td>
</tr>
<tr>
<td>337, 339, 343</td>
</tr>
</tbody>
</table>

DNA samples in set 1 were prepared from 0.5 g tissue, and in set 2 from 2 g. + indicates that the sample gave a positive result in at least two reactions performed on different occasions; ++ indicates a strong positive result.
titre. The uninfected control animals did not show a detectable titre (mean titre always 0).

3.6. Lymphocyte responses against N. caninum tachyzoite extract

Lymphocytes isolated from the blood, lymph nodes or spleen showed very strong proliferative responses after incubation with ConA on all dates tested (results not shown). Peripheral blood lymphocyte responses to N. caninum tachyzoite extract (10 µg ml⁻¹) were detected on day 7 p.i. and on all other dates tested (results not shown, mean c.p.m.: 1927–2831). The uninfected control animals showed no proliferative responses above background at any time point. At day 70 p.i. (results not shown), responses to N. caninum oocyst extract (2 µg ml⁻¹) were detected in infected animals when compared with the uninfected animals.

Inguinal lymph node lymphocytes and splenic lymphocytes from the infected calves showed very strong responses to N. caninum tachyzoite extract (2 µg ml⁻¹) (Fig. 3) compared with the uninfected control calves. Bronchial and mesenteric lymph node lymphocytes showed low responses in the infected calves (Fig. 3) compared with the uninfected control calves.

3.7. Lymphocyte subpopulations

Flow cytometric analyses showed a lower percentage of T-cells (CD3⁺ cells) in spleen (P < 0.002), mesenteric (P < 0.033) and bronchial (P < 0.219, not shown) lymph nodes of infected animals, but not in inguinal lymph nodes (P < 0.991, not shown), compared with the uninfected control animals (Fig. 4). No differences were seen for percentages of T-helper cells (CD4⁺), cytotoxic T-cells (CD8⁺), IL2 receptor+ cells, γδ T-cells (γδ TcR⁺), B-cells (B–B4⁺), monocytes and macrophages (CD14⁺) between infected and uninfected animals (Fig. 4).

4. Discussion

Until now, infection with N. caninum had been demonstrated to occur in two ways. First, oral infection through ingestion of N. caninum tissue cysts [15] which is unlikely in herbivores, and secondly, transplacental infection of the foetus during pregnancy (vertical transmission) [29–32]. However, horizontal transmission also appears to be necessary for the introduction of new infections in the herd [31–34]. Until the recent discovery of the oocyst stage [9], environmental transmission of N. caninum in cattle was unclear, and horizontal transmission had not been demonstrated. Results of the present study confirm the potential for horizontal transmission by oral ingestion of N. caninum oocysts as a third mode of infection. Experimental infection of cattle with N. caninum oocysts induced measurable cellular and humoral immune responses, in-

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Table 3
Antibody titres to Neospora caninum in calves fed N. caninum oocysts determined by IFAT and the N. caninum agglutination test

<table>
<thead>
<tr>
<th>Calf</th>
<th>IFAT⁺</th>
<th>NAT⁺</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Day 14</td>
<td>Day 28</td>
</tr>
<tr>
<td>334</td>
<td>100</td>
<td>200</td>
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<tr>
<td>335</td>
<td>200</td>
<td>200</td>
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<tr>
<td>338</td>
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<tr>
<td>342</td>
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<tr>
<td>344</td>
<td>200</td>
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<td>345</td>
<td>&lt; 25</td>
<td>&lt; 25</td>
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<tr>
<td>346</td>
<td>800</td>
<td>1600</td>
</tr>
<tr>
<td>337,339,343</td>
<td>&lt; 25</td>
<td>&lt; 25</td>
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</table>

⁺No calves had any antibodies to N. caninum in 1:25 dilution on the day of oocyst feeding.
Fig. 1. Mean ± 1 S.D. IgG1 (a), IgG2 (b), and IgM (c) antibodies (absorbance) against *Neospora caninum* tachyzoite extract of seven animals infected with *N. caninum* oocysts and three uninfected control animals.
indicating exposure to parasite antigens. Moreover, parasite DNA was detected by PCR in the brain and spinal cord of infected but not uninfected calves. That *N. caninum* was not always detected in repeated reactions with the same samples indicates the presence of very low numbers of parasites. In addition, PCR analyses performed on different portions from the same tissue were not always positive, indicating that parasites are not uniformly distributed in these tissues.

Inoculation with *N. caninum* oocysts resulted in a strong cell proliferation to a crude *N. caninum* tachyzoite extract as early as 1 week p.i. Cells taken from uninfected control animals did not show any proliferation. Proliferative lymphocyte responses to *N. caninum* tachyzoite extract were also recorded in calves s.c. inoculated with *N. caninum* tachyzoites [16, 35], and splenocytes from mice infected with *N. caninum* tachyzoites proliferated in vitro to *N. caninum* tachyzoite extract [36].

The calves which were seronegative to *N. caninum* prior to oral inoculation seroconverted as early as 2–4 weeks p.i., while the uninfected control calves remained seronegative during the

![Figure 2](image2.png)

**Fig. 2.** Mean titres ± 1 S.D. of antibody (IgG) against recombinant tachyzoite antigen in sera from seven calves infected with *N. caninum* oocysts and in sera from three uninfected control animals.

![Figure 3](image3.png)

**Fig. 3.** Mean proliferation ± 1 S.D. of mesenteric, bronchial and inguinal lymph node lymphocytes and spleen lymphocytes of seven animals infected with *Neospora caninum* oocysts and three uninfected control animals to *N. caninum* tachyzoite extract.
Fig. 4. The mean percentage ± 1 S.D. of different cell subpopulations in the mesenteric lymph node (a) and in the spleen (b) of seven animals infected with *N. caninum* oocysts and of three uninfected control animals. * indicates a significant difference (*P* < 0.05) between infected and control animals.
entire study. With the exception of calf 345, the results were similar regardless of the assay used. In comparison, calves inoculated s.c. or orally with *N. caninum* tachyzoites seroconverted 3 and 5 weeks p.i., respectively [17, 35]. Interestingly, the anti-*Neospora* IgG1 and IgG2 remained at a plateau, indicating active infection with ongoing antigenic stimulus. In one infected animal no anti-*N. caninum* antibodies were demonstrated except by NAT (although parasite DNA was detected in the brain and spinal cord by PCR), perhaps indicating that animals can be infected without mounting a demonstrable serum antibody response against *N. caninum*. More work will be necessary to clarify the possible heterogeneity in antibody responses in cattle.

Preliminary results in this study indicate a lower percentage of T-cells in the spleen, mesenteric and bronchial lymph nodes from calves infected with *N. caninum* oocysts compared with non-infected calves. These results further demonstrate that oral inoculation of the calves with canine-derived oocysts induced systemic changes in the animal’s immune system. The significance of this finding awaits collection of a more extensive data set. Very little is known concerning the role of different cell subpopulations and cytokines in the bovine immune response against *N. caninum*. High levels of IFN-γ have been shown in calves infected with *N. caninum* tachyzoites [35]. The role of IFN-γ in mediating resistance against neosporosis has been established in mice, where it was demonstrated that neutralisation of IFN-γ at the time of infection results in mortality of the infected mice [36].

References

[10] Lindsay DS, Dubey JP, Duncan RB. Confirmation that the dog is a definitive host for *Neospora caninum*. Vet Parasitol 1999;82:327–33.


