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## Oral *Neospora caninum* inoculation of neonatal calves

A. Uggl<sup>a,\*</sup>, S. Stenlund<sup>b</sup>, O.J.M. Holmdahl<sup>a</sup>, E.-B. Jakubek<sup>a</sup>, P. Thebo<sup>a</sup>,  
H. Kindahl<sup>b</sup>, C. Björkman<sup>c</sup>

<sup>a</sup>Department of Parasitology, National Veterinary Institute and Swedish University of Agricultural Sciences, PO Box 7073, SE-750 07 Uppsala, Sweden

<sup>b</sup>Department of Obstetrics and Gynaecology, Swedish University of Agricultural Sciences, PO Box 7039, SE-750 07 Uppsala, Sweden

<sup>c</sup>Department of Ruminant Medicine and Veterinary Epidemiology, Swedish University of Agricultural Sciences, PO Box 7019, SE-750 07 Uppsala, Sweden

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### Abstract

Four calves born to cows seronegative for *Neospora caninum* were dosed orally within 6 h after birth with tachyzoites of the bovine *N. caninum* Nc-SweB1 isolate added to colostrum. Two of the calves were dosed via stomach tube and two by feeding bottle. The latter two calves showed transient fever and passed blood-stained diarrhoea 1–2 weeks after inoculation. From 5 weeks after inoculation they developed a significant antibody response which remained high until the calves were euthanised and necropsied at 15 and 19 weeks after inoculation, respectively. The two calves inoculated by stomach tube showed no clinical signs and they remained seronegative throughout the study. At necropsy of the seropositive calves, no pathological lesions were seen, and parasites were not detected by immunohistochemistry. *Neospora caninum* was not re-isolated in cell culture from the brains of the seropositive calves; however, *N. caninum* DNA was detected in brain from both of them by PCR. The data suggest that oral infection of *N. caninum* via colostrum might be a possible route of vertical transmission in newborn calves, in addition to transplacental infection. © 1998 Australian Society for Parasitology. Published by Elsevier Science Ltd. All rights reserved.

**Keywords:** *Neospora caninum*; Transmission; Oral inoculation; Cattle; Colostrum

### 1. Introduction

The coccidian parasite *Neospora caninum* is closely related to *Toxoplasma gondii* and has an intermediate host range including dogs and cattle, but a definitive host capable of shedding oocysts has not been identified [1]. Consequently, since the

complete life-cycle of the parasite has not yet been elucidated, not all aspects of the epidemiology of *N. caninum* infections are known.

In cattle it appears that the major route of transmission of *N. caninum* is transplacental, resulting in the birth of congenitally infected progeny [1]. Such transmission can take place repeatedly during subsequent pregnancies in latently infected cows [2]. In some investigations of neosporosis outbreaks in cattle herds there has been evidence suggesting an external source of infection, although oral infection of possible oocysts in the environment or

\* Corresponding author. Tel: +46-18 67 40 00; Fax: +46-18 30 91 62; e-mail: arvid.uggla@sva.se

through contact with infected tissues has not been demonstrated [3, 4].

With *T. gondii*, transmission via milk has been shown to be a possible route of infection in mice [5, 6] and probably also in humans [7–9]. However, there are no reports on galactogenic transmission of *N. caninum* [1], although this mode of infection can be hypothesised [10]. The aim of the present study was to investigate the potential of *N. caninum* tachyzoites to infect neonatal calves when given orally with colostrum.

## 2. Materials and methods

### 2.1. Animals

Two female (Nos 1 and 4) and two male (Nos 2 and 3) calves of the Swedish Red and White breed were included in the study. They were born at full term to four cows seronegative to *N. caninum* as demonstrated by ELISA [11]. The births were induced by i.m. injection of 20 mg dexamethasone daily for 2 consecutive days approximately 3 weeks before expected date of delivery. The calvings, which occurred within 10 days of one another, were supervised, and the calves were prevented from sucking their mothers. After birth they were immediately separated from their dams and placed in individual pens. The calves were fed homologous colostrum with added *N. caninum* (see below) within 6 h after birth (day 0) and 2 l of fresh milk 2–3 times daily from day 1 until weaning at 8 weeks of age. Hay and commercial pelleted calf fodder ad libitum were introduced from day 1.

The calves were observed daily for clinical signs, appetite and faecal condition. Rectal temperatures were recorded daily during the first 3 weeks and twice weekly thereafter until termination of the experiment after 15 weeks (19 weeks for calf 3). Blood samples were collected by jugular vein puncture immediately after delivery (precolostral samples) and then at weekly intervals. Serum was separated off and stored at  $-20^{\circ}\text{C}$  until analysed for *N. caninum* antibodies by an iscom ELISA as described by Björkman et al. [11]. Calves 1 and 2 were removed from the study after 15 weeks,

whereas calves 3 and 4 were euthanised and necropsied at 19 and 15 weeks of age, respectively.

### 2.2. Inoculum

The bovine *N. caninum* isolate Nc-SweB1 was cultivated on Vero cells as described by Stenlund et al. [12]. Tachyzoites were harvested by treating the cell layers with 0.1% trypsin at  $37^{\circ}\text{C}$  for 5–10 min. A few millilitres of RPMI medium were added and the suspension centrifuged at 1000 g, whereafter the pellet was resuspended in RPMI medium at  $37^{\circ}\text{C}$  [12]. The parasites were counted in a haemocytometer and transported approximately 500 m in an insulated box at  $37^{\circ}\text{C}$  to the animal house where they were added to 500 ml of newly milked colostrum at  $37^{\circ}\text{C}$  from the calf's mother. The parasite–colostrum suspension was given to calves 1 and 2 via gastric tubes and to calves 3 and 4 with feeding bottles. Approximately  $3\text{--}10 \times 10^7$  tachyzoites were given to each calf. An additional 500–1000 ml of colostrum was given to the calves after the parasite suspension. All doses were given within 6 h of birth of the calves.

### 2.3. Post-mortem procedures

After euthanasia of calves 3 and 4 by means of an i.v. barbiturate overdose, they were immediately necropsied and their brains removed aseptically. Specimens from cerebrum, eye, cervical, thoracic and lumbosacral spinal cord, heart, skeletal muscle (thigh), lung, liver, spleen, kidney, abomasum, jejunum, caecum, colon, thymus, tonsils, ileocaecal and bronchial lymph nodes, and testis/ovary were fixed in 10% buffered formalin, embedded in paraffin, sectioned at  $4\ \mu\text{m}$  and stained by H & E according to standard procedures. Sections from seven areas of the brain and three regions of the spinal cord were also subjected to immunohistochemical labelling using a rabbit anti-*N. caninum* serum in the peroxidase anti-peroxidase (PAP) system according to Lindsay and Dubey [13].

Attempts to re-isolate *N. caninum* from the two brains were performed essentially as described by Stenlund et al. [12]. In addition, samples from different parts of the CNS were kept at  $-70^{\circ}\text{C}$  until analysed by PCR for *N. caninum* DNA. For the

PCR, 0.3-g portions were extracted as described [14]. In order to enhance the sensitivity, a nested PCR was modified from the method described by Holmdahl and Mattsson [14]. The external primers F6 and 5.8B, 5'-CAG GTC TGT GAT GCC C-3' and 5'-TCG CGT TTT GCT GCG TTC TTC-3', respectively, were complementary to regions of the rDNA unit (18S and 5.8S), and used in the first PCR. The internal primers PN3 and PN4, 5'-TAC TAC TCC CTG TGA GTT G-3' and 5'-TCT CTT CCC TCA AAC GCT A-3', respectively, specific for *N. caninum*, were complementary to the internal transcribed region 1 (ITS1), and were used in the second PCR. In both PCRs the samples comprised 3  $\mu$ l. The samples for the second reaction were handled in a room separate from the first reaction and were trapped between layers of oil during the pipetting in order to minimise the risk of contamination. The 50- $\mu$ l reaction mixtures used for each step contained 10 mM-Tris-HCl (pH 8.3), 50 mM-KCl, 2 mM-MgCl<sub>2</sub>, 200  $\mu$ M of each deoxynucleotide, 0.8 U of Ampli Taq DNA polymerase (Perkin-Elmer, U.S.A.) and sample. Primer concentrations were 0.1  $\mu$ M and 0.4  $\mu$ M in reactions 1 and 2, respectively.

Initially the samples were heated for 2.5 min at 94°C; thereafter each cycle consisted of a 30-s denaturation at 94°C, 40 s at the annealing temperature of 50°C, followed by a 1-min extension at 72°C. Amplification was performed with F6 and 5.8B over 25 cycles followed by a final extension at 72°C for 3 min. In the second PCR the annealing temperature was increased to 54°C, extension decreased to 30 s and the amplification was performed with PN3 and PN4 over 30 cycles, as above. *Neospora caninum* DNA from cell culture grown Nc-SweB1 organisms [12] was used as positive control, and water as negative control.

Eight-microlitre aliquots of reaction mixtures were analysed by 1.5% agarose gel electrophoresis and the products stained with ethidium bromide and visualised under u.v. light.

### 3. Results

#### 3.1. Clinical observations

Rectal temperatures of calves 1 and 2 remained within the range 38.5–39.5°C throughout the obser-

vation period. Calf 3 had a temperature of 39.7°C at 3, 8 and 10 days p.i., otherwise a maximum temperature of 39.5°C. Calf 4 had 39.8°C at 2 days p.i., 39.9°C at 9 days p.i. and 39.7°C at 10 and 18 days p.i., otherwise a maximum of 39.5°C.

Calf 3 had blood-stained diarrhoea 15 and 16 days p.i. and showed diarrhoea not containing blood occasionally after that. Calf 4 had blood-stained diarrhoea 13 days p.i. Apart from this, no specific clinical signs were observed.

#### 3.2. Serological response

The *N. caninum* antibody reactions are shown in Figure 1. Calves 1 and 2 were seronegative, with absorbance values well below the cut-off limit of 0.2 absorbance units throughout the 15-week observation period. However, in calves 3 and 4 a significant rise in antibody levels was observed from 4 to 5 weeks p.i. and they remained at high levels until the termination of the study.

#### 3.3. Demonstration of *N. caninum*

Neither gross nor histological pathologic changes were seen at autopsy of calves 3 and 4. No *N. caninum* organisms were detected by immunohistochemical analysis.

Growth of *N. caninum* was not detected in the cell cultures inoculated with brain tissue material from calves 3 and 4. However, by PCR, *N. caninum* DNA was repeatedly demonstrated in one sample each from the brains of calves 3 and 4 (Fig. 2). Both positive PCR samples originated from the hippocampus region of the cerebrum.

### 4. Discussion

The present study has provided evidence that galactogenic transmission of *N. caninum* is possible in neonatal calves. Two newborn calves that were inoculated via feeding bottle with *N. caninum* organisms mixed with colostrum reacted with seroconversion, and in their brains, *N. caninum* DNA was demonstrated by PCR. The study thus also illustrated the virtues of PCR as compared with immunohistochemistry and isolation experiments

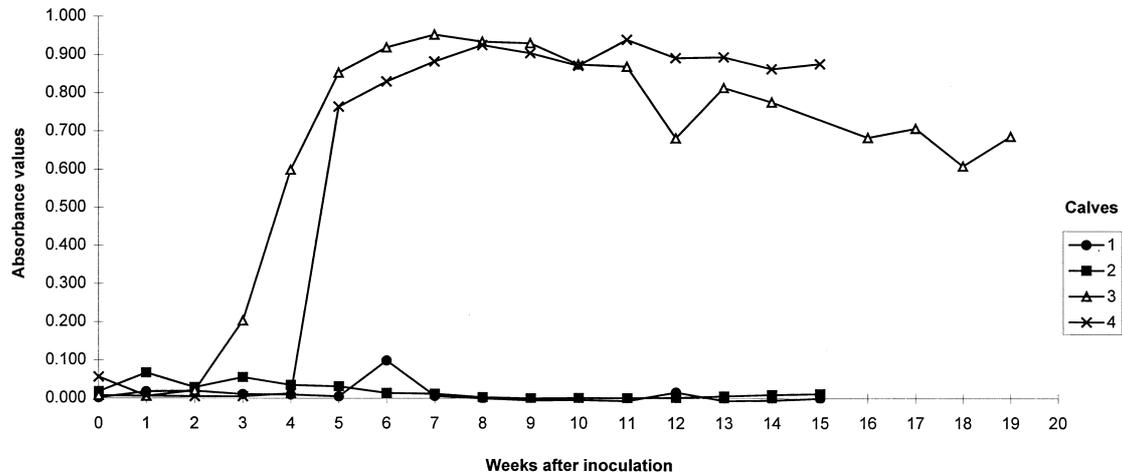


Fig. 1. Serological response, as measured by *Neospora caninum* iscom ELISA, of four calves inoculated orally as newborns with *N. caninum* tachyzoites mixed with colostrum.

for the demonstration of *N. caninum* when present in small numbers. The antibody responses recorded in the two calves were of significant magnitude and commenced at a similar time after the inoculation. The antibody levels then remained at a plateau, indicating active infection with ongoing antigenic stimulus obviously due to an active infection. Although the experiment was not performed under gnotobiotic conditions, we consider it unlikely that there was any other source of *N. caninum* infection in the two calves other than the inoculation performed. The two calves that remained seronegative

were housed in the same animal room and were fed the same fodder as the calves that seroconverted.

*Neospora caninum* is closely related to *T. gondii* [15, 16], where the knowledge of transmission and general epidemiology has a longer history. Galactogenic transmission of *T. gondii* has been demonstrated experimentally in mice [5, 6], and *T. gondii* tachyzoites in unpasteurised goat milk have been incriminated as the source of human toxoplasmosis on repeated occasions [7–9]. Furthermore, *T. gondii* has been isolated from colostrum of humans [17]. Although *T. gondii* may survive in milk for up to 6 days [18], it has been believed that tachyzoites present in milk should not be able to survive the gastric juices when passing through the stomach [19]. However, in the digestive tract of the newborn, the production of hydrochloric acid and enzymes is low [20] and the proteolytic activity is further minimised by trypsin inhibitors present in the colostrum [21]. These conditions in the digestive tract of neonatal calves may permit *N. caninum* organisms to retain their infectivity if ingested with colostrum during the first few hours of the calf's life.

In our experiment the inoculum consisted of a crude cell culture harvest containing free *N. caninum* tachyzoites as well as organisms still enclosed within Vero cells. Therefore, it could also be possible that the parasites enclosed in their host cells

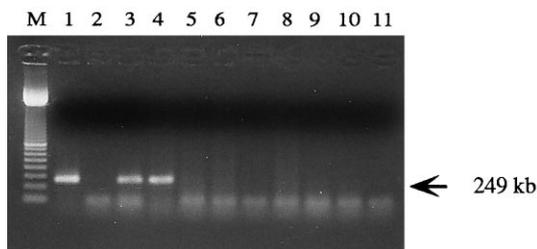


Fig. 2. Agarose gel electrophoresis of the products obtained by PCR using DNA extracted from *Neospora caninum* (positive control, lane 1), water (negative control, lanes 2 and 11), hippocampus of calves 3 and 4 (lanes 3 and 4, respectively), parietal cortex of calves 3 and 4 (lanes 5 and 6), basal ganglia of calves 3 and 4 (lanes 7 and 8) and cerebellum of calves 3 and 4 (lanes 9 and 10). A 100-bp ladder (M) from Amersham Pharmacia Biotech was used as molecular size marker.

would better resist passage through the abomasum than free tachyzoites and thereby retain their infectivity when reaching the small intestine. One reason why only the calves inoculated via feeding bottle became infected could be that, by this approach, the oesophageal groove was stimulated to direct the milk straight into the abomasum. Inoculation by gastric tube might have caused most of the inoculated volume to end up in the rumen, where the parasites could have been compromised. Another hypothesis is that by using bottle inoculation the parasites were allowed to enter the host by penetration of the buccal or pharyngeal mucosa, as demonstrated with *T. gondii* in mice [6].

The clinical signs observed in the two calves that seroconverted, namely a slight febrile response and a transient blood-stained diarrhoea, were mild and non-specific. No clinical signs at all were observed in the two calves that remained seronegative. If the intestinal mucosa was the entrance-gate for the parasites, one may speculate that the diarrhoea could have been the result of an initial local proliferation of the parasites in the intestinal mucosa, similar to that observed after oral *T. gondii* infection in different animal hosts [22]. However, no analyses were made with regard to other possible causes of the diarrhoea in the two calves.

The full epidemiology of *N. caninum* in cattle remains to be elucidated. That there should be an additional source of transmission of *N. caninum* other than the transplacental route can be presumed. Although the present study has provided evidence that transmission via milk is possible in newborn calves, the issue as to whether galactogenic spread would provide any significant contribution to the distribution of *N. caninum* among cattle will be clarified only after further experimental and epidemiological studies.

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