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Characterization of a Swedish bovine isolate of *Neospora caninum*

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Abstract The brain of a stillborn calf, seropositive to *Neospora caninum* and born to a seropositive cow, was homogenized and cultured on Vero cells, where growth of *Neospora*-like tachyzoites was detected after 8 weeks. The ultrastructural features of the new isolate (Nc-SweB1) corresponded to those of previously published *Neospora* isolates. In indirect immunofluorescence tests, antigens on Nc-SweB1 tachyzoites were recognized by antibodies raised to a canine *N. caninum* isolate (Nc-1) but not by antibodies to *Toxoplasma gondii*, *Sarcocystis cruzi, S. tenella, Eimeria alabamensis, Babesia divergens*, or *B. motasi*. Immunoblot analyses revealed no major antigenic difference between Nc-SweB1 and Nc-1, whereas several differences were seen between Nc-SweB1 and protozoa related to *N. caninum*. The sequences of 16S-like rRNA and the internal transcribed spacer 1 of Nc-SweB1 revealed complete homology with corresponding sequences of two canine *N. caninum* isolates. Thus, no dissimilarity between Nc-SweB1 and the canine isolates was found, confirming that Nc-SweB1 is *N. caninum* and suggesting that *Neospora*-like organisms isolated from cattle are indeed *N. caninum*.

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Introduction

The cyst-forming coccidian parasite *Neospora caninum* (Dubey et al. 1988a) belongs to the family Sarcocystidae of the phylum Apicomplexa (Holmdahl et al. 1994). The complete life cycle of *N. caninum* has not yet been established; however, a likeness to *Toxoplasma gondii*-suggests a carnivore as the definitive host (Dubey and Lindsay 1993). The parasite was first reported to cause paralysis in young dogs (Bjerkås et al. 1984; Dubey et al. 1988a). A few years later, *Neospora*-like organisms were shown to be an important cause of abortion in cattle (Thilsted and Dubey 1989; Anderson et al. 1991).

The presence of the parasite in Sweden has been established in dogs (Uggla et al. 1989; Björkman et al. 1994b) as well as in cattle (Holmdahl et al. 1995; Björkman et al. 1996b). Since the discovery of *N. caninum,* a few successful isolations of the parasite have been reported from dogs in the United States (Dubey et al. 1988b) and in England (Barber et al. 1995). Isolates of *Neospora*-like parasites from cattle, however, have been described only in the United States (Conrad et al. 1993).

This paper reports the successful isolation of *N. caninum* from a stillborn Swedish calf and its subsequent characterization by electron microscopy, indirect fluorescent antibody test, Western blotting and molecular biological techniques, comparing it with the canine *N. caninum* reference isolate Nc-1 and other closely related parasites. The new isolate was designated Nc-SweB1. In some studies on bovine neosporosis it has not been ruled out that *Neospora* isolates from dogs and cattle may be of different species (Conrad et al. 1993). We therefore wanted to investigate specifically whether the new bovine isolate differed in any significant way from canine *N. caninum* isolates.

Materials and methods

Isolation and cultivation

In March 1995 a stillborn calf delivered by a N*eospora caninum*-seropositive cow was brought to the laboratory. It arrived within 72 h of delivery after having been kept at approximately 2 °C. Samples were collected for histological and immunohistochemical examination (Lindsay and Dubey 1989), and cardiac blood was collected for serology (Björkman et al. 1997).

The brain was removed aseptically and immersed in phosphate-buffered saline at pH 7.4. It was homogenized, digested with 0.25% trypsin in PBS at 37 °C for 30 min, and centrifuged at 3,000 *g* for 10 min. The pellet was resuspended in an isotonic 30% Percoll solution (Pharmacia LKB Biotechnology, Uppsala, Sweden) and centrifuged at 3,000 *g* for 20 min to remove debris and fat. The pellet was washed twice with PBS; cultivated on a 24-h monolayer of Vero (African green monkey kidney) cells with RPMI 1640 medium supplemented with 60 mg penicillin/ml, 50 mg dihydrostreptomycin sulfate/ml, 4 m*M* L-glutamine, and 2% fetal calf serum (FCS; National Veterinary Institute, Uppsala, Sweden); and incubated at 37 °C in a 5% CO_2/95% air environment.

Medium was changed twice weekly. To keep the total amount of cultured cells to a minimum, subcultures were made at 10- to 11-day intervals. The cultures were scanned every week.

After the detection of parasites, cultivation on Vero cells was continued using RPMI 1640 medium supplemented as described above. During the first 7 weeks, no fresh Vero cells were added. At between 7 and 11 weeks, Vero cells were added at an increasing rate, and after 11 weeks the parasites were passaged every 7–10 days. The isolate was designated Nc-SweB1. The Nc-1 isolate (Dubey et al. 1988b) was maintained in Vero-cell cultures similarly to the Nc-SweB1 isolate, but the parasites were passaged every 5–7 days.

Production of polyclonal antisera

Heavily infected cell cultures were treated with 0.25% trypsin in phosphate buffer with 0.02% Tritriplex III (pH 7.4) at 37 °C for 5 min and centrifuged at 2,000 *g* for 10 min. The pellet was resuspended in an isotonic 30% Percoll solution and centrifuged at 2,000 *g* for 10 min, after which the resulting pellet was washed twice with PBS.

Four New Zealand White rabbits, which were seronegative by indirect fluorescent antibody test (IFAT) to *Toxoplasma gondii* and *N. caninum*, were used for production of antisera. Two of the rabbits were injected intramuscularly with 10^4 tachyzoites of Nc-SweB1 on days 0 and 10. The other two were injected with 10^7 killed tachyzoites and Freund's adjuvant (Sigma Chemical Company, St. Louis, USA) on days 0, 21, and 35. Blood was collected, and serum was prepared at 9 weeks after the first inoculation, when the *N. caninum* IFAT titres were 1:10,240.

Ultrastructure studies

Heavily infected cell cultures were treated with trypsin as described above. The resulting pellet was fixed in 2% glutaraldehyde in sodium cacodylate buffer and postfixed in 1% osmium tetroxide in distilled water for 1 h at 4 °C. After being dehydrated in an ethanol series via propylene oxide, the pellet was embedded in Agar-100 resin plastic (Agar Scientific LTD, Stansted, Essex, UK). Ultrathin sections were stained with uranyl acetate and lead citrate (Reynolds 1963) and examined in a Philips 420 transmission electron microscope operating at 60 kV.

Indirect fluorescent antibody test

The parasites were used as the antigen in an IFAT, which was performed essentially as described by Dubey et al. (1988b). They were grown as described above, but for the last two passages, horse serum was included in the medium instead of FCS. Purified parasites were diluted to 5×10^7/ml with PBS and dried onto 18-well ethanol-washed microscope slides.

Sera from dogs, cattle, rabbits, or sheep inoculated with *N. caninum* or other closely related apicomplexan parasites were used as primary antibodies (Table 1). The sera were diluted in 2-fold serial dilutions in PBS, starting from 1:20 (dog, rabbit, and sheep sera) or from 1:320 (cattle sera). Sera from animals infected with *N. caninum* were included in each series of analysis as positive controls. Fluorescein isothiocyanate (FITC)-labeled swine anti-rabbit IgG (Dakopatts, Copenhagen, Denmark), rabbit anti-sheep IgG (Dakopatts), rabbit anti-dog IgG (Sigma), and rabbit anti-cattle IgG (Sigma) antibodies were used as conjugate. The sera were considered negative if no fluorescence was detected at serum dilutions of 1:40 (dog, rabbit, and sheep sera) or 1:640 (cattle sera).

Western-blot analysis

Western blot analysis was performed under nonreducing conditions essentially as described by Björkman et al. (1994a). The Nc-SweB1 and Nc-1 isolates, purified on Percoll as described above, and Vero cells were probed with rabbit and cattle sera.

Rabbit sera were obtained from animals immunized with tachyzoites of Nc-SweB1, Nc-1, or *T. gondii* (RH strain) or with cystozoites of *Sarcocystis cruzi.* Serum from a rabbit found to be seronegative to these parasites by IFAT was used as the negative control. The sera were diluted 1:1,000 in PBS with 0.05% Tween-80 (PBS-T). Alkaline phosphatase (AP)-conjugated swine antibodies to rabbit IgG (Dakopatts) were used as the secondary antibody.

Bovine sera were obtained from animals inoculated with tachyzoites of Nc-1 and *T. gondii*, cystozoites of *S. cruzi*, oocysts of *Eimeria alabamensis*, or piroplasms of *Babesia divergens* and from the naturally infected calf from which Nc-SweB1 was isolated. As the negative control we used serum from a calf found to be seronegative to these parasites. The bovine sera were diluted 1:10 in PBS-T. A mixture of monoclonal mouse antibodies to cattle IgG (Svanova, Uppsala, Sweden) and AP-conjugated rabbit antibodies to mouse Ig (Dakopatts) was used as the conjugate.

Solid-phase sequencing

The sequences of 16S-like ribosomal RNA (rRNA) and the internal transcribed spacer 1 (ITS1) of Nc-SweB1 were determined by solid-phase sequencing and were computer-aligned with corresponding sequences of the Nc-1 and Nc-Liv isolates as described by Barber et al. (1995).

Table 1. Results of application of antibodies in the IFAT employing tachyzoites of the isolate Nc-SweB1 as the antigen

Rabbit anti-Nc-SweB1	Positive
Cattle anti-Nc-1	Positive
Dog anti-*Neospora caninum*[a]	Positive
Rabbit anti-Nc-1	Positive
Sheep anti-Nc-1	Positive
Cattle anti-*Toxoplasma gondii*, RH strain	Negative
Dog anti-*T. gondii*[a]	Negative
Rabbit anti-*T. gondii* RH strain	Negative
Cattle anti-*Sarcocystis cruzi*	Negative
Rabbit anti-*S. cruzi*	Negative
Rabbit anti-*S. tenella*	Negative
Cattle anti-*Babesia divergens*	Negative
Sheep anti-*B. motasi*	Negative
Cattle anti-*Eimeria alabamensis*	Negative

[a]Natural infection; all other sera were obtained from experimentally immunized animals

Results

Isolation and in vitro growth characteristics

No lesion typical of *Neospora caninum* infection was seen in histological preparations of brain, spinal cord, heart, lung, liver, kidney, spleen, lymph node, or skeletal muscle, and no parasite could be detected by immunohistochemistry. The calf was found to be seropositive to *N. caninum* by IFAT.

After 8 weeks, parasites were visually detected in the flasks inoculated with brain tissue from the stillborn calf. They were first seen as weakly motile extracellular tachyzoites in holes in the layer of Vero cells. In the adjacent Vero cells we observed intracellular parasites appearing, singly or in small clusters. Few large clusters were seen in this first phase. The size and appearance of the tachyzoites were in accordance with those of the Nc-1 isolate, but the growth rate was initially very slow in comparison with that of the established canine isolate. For many weeks, no fresh Vero cells were added to the cultures. At around 2 months after detection, the parasites had accelerated in growth and it became necessary to add fresh Vero cells. After 11 weeks, i.e., at 19 weeks after inoculation of the flasks, the parasites were passaged every 7–10 days. The isolate has been kept in culture since March 1995, for more than 40 generations, and has been frozen and thawed with no loss of viability.

Ultrastructure studies

Electron microscopy revealed parasites of approximately 5×1.3 μm containing 4–16 anterior rhoptries, but no posterior rhoptry. There were many anterior micronemes and posterior as well as anterior electron-dense bodies. A posterior residual body was often noted, whereas micropores were rare. Other apicomplexan structures such as polar rings, posterior polar rings, microtubules, and conoids were present. The tachyzoites were contained in vacuoles of the host cells and were seen to divide by endodyogeny; no intranuclear parasite could be found.

Indirect fluorescent antibody test

Antigens on tachyzoites of the Nc-SweB1 isolate were recognized by cattle, dog, rabbit, and sheep polyclonal antisera raised to the Nc-1 isolate. Antibodies to *Toxoplasma gondii*, *Sarcocystis cruzi, S. tenella, Eimeria alabamensis, Babesia divergens,* and *B. motasi* did not react with the organisms (Table 1).

Western-blot analysis

Rabbit anti-Nc-1 serum and rabbit anti-Nc-SweB1 serum recognized several antigens with molecular weights of between 16 and 91 kDa in both the Nc-SweB1 and Nc-1 isolates. The most heavily stained bands had approximate molecular weights of 28, 31, 40, 83, and 91 kDa (Fig. 1). A band at 40 kDa was faintly stained by anti-*T. gondii* serum, whereas anti-*S. cruzi* and negative control serum exhibited negligible binding (Fig. 1). Two weakly stained bands at 28 and 40 kDa were seen when antisera to both isolates were applied on Vero-cell preparations (results not shown).

Serum from the calf experimentally infected with Nc-1 tachyzoites and serum from the naturally infected calf also recognized the same antigenic components in the two *Neospora* isolates at approximately 24–75 kDa (Fig. 2). Dominant antigens were found at 40, 60, 65, and 75 kDa. The bands were markedly more faintly stained when serum from the naturally infected calf rather than serum from the experimentally infected calf was used as the probe. Bovine antisera to the four closely related parasites exhibited no binding at all to the Nc-SweB1 or Nc-1 isolate. No binding to the host-cell antigen preparations by any of the bovine antisera was seen.

Solid-phase sequencing

The 16S-like rRNA and ITS1 sequences of Nc-SweB1 were determined with few ambiguities. The alignment of the 16S-like rRNA and ITS1 revealed complete homology between the three *Neospora* isolates studied. However, a large number of sequence differences arose when the ITS1 sequence of Nc-SweB1 was compared with the corresponding sequence of *T. gondii*.

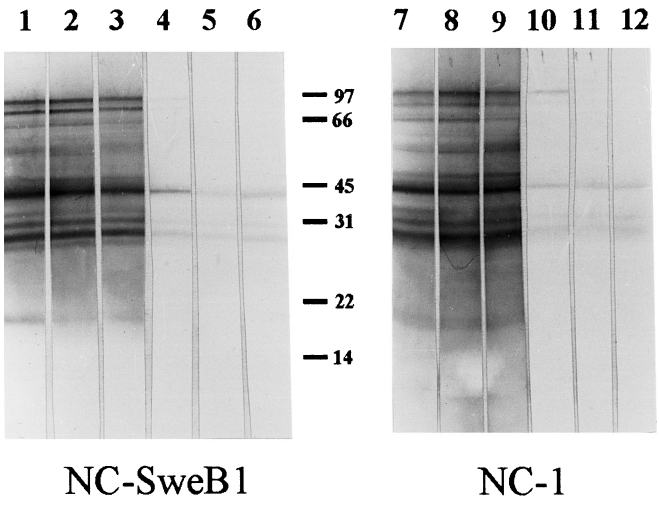

Fig. 1 Western-blot analysis of proteins from *Neospora* isolates Nc-SweB1 (*lanes 1–6*) and Nc-1 (*lanes 7–12*) separated by SDS-PAGE on a 12% polyacrylamide gel under non-reducing conditions. *Lanes 1*, *2*, *7*, *and 8* were probed with rabbit anti-Nc-SweB1; *lanes 3 and 9*, with rabbit anti-Nc-1; *lanes 4 and 10*, with rabbit anti-*Toxoplasma gondii*; *lanes 5 and 11*, with rabbit anti-*Sarcocystis cruzi*; and *lanes 6 and 12*, with negative control serum

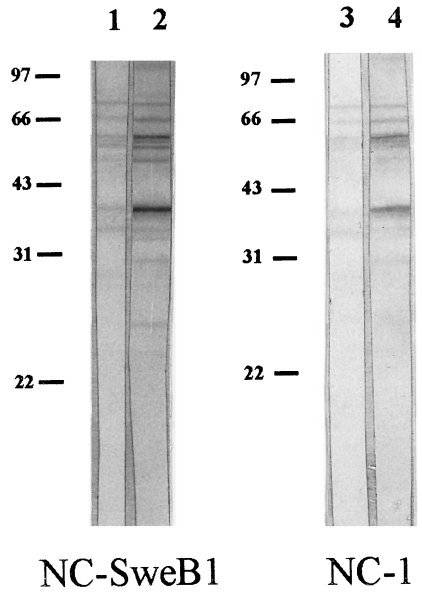

Fig. 2 Western-blot analysis of proteins from *Neospora* isolates Nc-SweB1 (*lanes 1, 2*) and Nc-1 (*lanes 3, 4*) separated by SDS-PAGE on a 12% polyacrylamide gel under non-reducing conditions. *Lanes 1 and 3* were probed with serum from the naturally infected calf from which Nc-SweB1 was isolated and *lanes 2 and 4*, with cattle anti-Nc-1 serum

Discussion

Few successful isolations of *Neospora* from cattle have been reported thus far, although many attempts have been made (Conrad et al. 1993). Conrad et al. (1993) suggest that an important prerequisite for success in isolation is minimal autolysis of the host tissue. Our results support this theory, since the calf from which the present isolation was made was relatively fresh having been estimated not to have been dead for more than 3–4 days at the time of autopsy and inoculation of cell cultures. Moreover, most of this time it had been kept at a temperature of approximately 2 °C. Lindsay et al. (1992) reported a survival time of 14 days for *Neospora caninum* tissue cysts kept at 4 °C.

Transplacental infection appears to be a dominant mode of transmission of *Neospora* in cattle (Björkman et al. 1996). The present calf originated from a herd in which 50% of all female animals were seropositive to *N. caninum*. It was born to a seropositive cow and was itself confirmed as being seropositive to *N. caninum*. *N. caninum* is most often found in the brain, but even there the parasite often occurs only sparsely (Barr et al. 1990). From the present calf, only small specimens of the CNS were taken for histological examination because most of the brain was used for the isolation attempts. Since we failed to find any parasites by immunohistochemistry, it is obvious that too few specimens, possibly from inappropriate parts of the CNS, were analyzed. Furthermore, immunohistochemistry has lately been found not to be a particularly sensitive diagnostic aid in neosporosis (McNamee et al. 1996). In another recently examined stillborn, seropositive calf from the same herd, numerous immunohistochemically stained sections of CNS had to be scrutinized before a few *N. caninum* organisms could be detected. Also in that case the occurrence of pathological lesions, such as gliosis, was minimal (E. Ågren, personal communication).

The only publication on isolation of bovine *Neospora* reports the detection of parasites at 15 and 34 days after inoculation of cell cultures (Conrad et al. 1993), whereas all canine isolates were detected earlier, e.g., Nc-Liv was detected after only 5 days (Barber et al. 1995). Nc-SweB1 organisms were not detected in the cell-culture flasks until 56 days after inoculation. A possible explanation is that the time lapsing between the death of the animal and inoculation of cell cultures may be crucial. Barber et al. (1995) performed autopsy and inoculation of cell cultures directly after euthanizing a dog, whereas in our case more than 72 h passed between the death of the calf and inoculation. The number of viable parasites could have decreased by then. Furthermore, that we could not find parasites by immunohistochemistry suggests a low number of parasites in the brain. Apart from being detected late, the present parasite isolate also had a very slow growth rate initially. This corresponds to the appearance of the two bovine isolates reported by Conrad et al. (1993). It thus seems that the canine isolates may be more easily adapted to growth in cell culture, although the reason for this is not clear.

For further characterization of the isolate, morphological, immunological, and molecular biological methods were applied. The ultrastructural features of Nc-SweB1 corresponded well to those published for the canine Nc-1 and Nc-Liv isolates (Lindsay et al. 1993; Barber et al. 1995) and the bovine isolates (Conrad et al. 1993). This is in agreement with the findings of Jardine (1996), who claimed that no distinct morphological difference existed between *N. caninum* from dogs and *Neospora*-like organisms from cattle.

Tachyzoites of the Nc-SweB1 isolate were recognized in the IFAT by serum from an *N. caninum*-infected dog and by sera from cattle, rabbit, and sheep inoculated with the Nc-1 isolate but not by antibodies *Toxoplasma gondii* or five other closely related protozoa. However, some cross-reactivity between *N. caninum* and *T. gondii* in naturally infected dogs has been reported by Trees et al. (1993). Some cross-reactivity has also been reported between *N. caninum* and the two *Babesia* species *B. gibsoni* and *B. canis* (Yamane et al. 1993). It is therefore noteworthy that in our tests, Nc-SweB1 did not show any cross-reactivity to two other *Babesia* species, *B. divergens* and *B. motasi*. By immunohistochemistry, Nc-1 has been shown by Conrad et al. (1993) to be antigenically distinct from *T. gondii* and *Sarcocystis cruzi*, which agrees with our findings that antisera to *T. gondii* and *S. cruzi* did not react with Nc-SweB1.

Western-blot analyses revealed no major difference between the two isolates Nc-SweB1 and Nc-1. Antisera raised to Nc-SweB1 and Nc-1 recognized antigens with

molecular weights of between 16 and 91 kDa in both isolates, the dominant antigens being of approximately 28, 31, 40, 83, and 91 kDa when rabbit sera were employed. Canine Nc-1 antigens have been analyzed in Western blots by Barta and Dubey (1992) and Bjerkås et al. (1994), who found four immunodominant antigens with molecular weights of between 16 and 46 kDa. Barber et al. (1995) reported ten dominant antigens in Nc-Liv and Nc-1 with molecular weights of between 17 and 95 kDa. Our results include faint or negligible binding to both isolates by antisera to *T. gondii* and *S. cruzi*. Bjerkås et al. (1994) obtained similar results in that antiserum to *S. cruzi* did not bind to Nc-1 antigen, whereas antiserum to *T. gondii* labeled several bands faintly. Also, when bovine sera were employed in our study, there was no major difference between Nc-SweB1 and Nc-1. However, the bovine sera recognized antigens of higher molecular weights than did the rabbit sera. When Paré et al. (1995) analyzed a bovine *Neospora* isolate in Western blots, they found no cross-reactivity between *Neospora* sp. and *T. gondii*, *Sarcocystis* sp., or *Eimeria* sp. at 1:100 serum dilutions. This concurs with our results showing that the bovine antisera to *T. gondii, S. cruzi*, and *E. alabamensis* did not contain cross-reactive antibodies to either Nc-SweB1 or Nc-1.

The complete homology observed in the 16S-like rRNA and ITS-1 sequences between the three *Neospora* isolates studied (Nc-SweB1, Nc-1, and Nc-Liv) strongly supports the contention that the newly characterized Nc-SweB1 isolate is *N. caninum.* Marsh et al. (1995) found no relevant difference when they compared sequences from 16S-like rRNA of canine and bovine *Neospora* isolates from the United States. In addition to that study, we now show that no difference exists in the ITS1 by comparison of corresponding sequences from one bovine and two canine *Neospora* isolates. Very few sequence differences have been reported in comparisons of the sequences of 16S-like rRNA between Nc-1 and *T. gondii* (Ellis et al. 1994; Holmdahl et al. 1994). A large number of differences arose in this study when we compared the ITS1 sequence of Nc-SweB1 with the corresponding sequence of *T. gondii*, which has also previously been shown for Nc-1 as well as for Nc-Liv (Barber et al. 1995). The homology seen in the ITS1 between Nc-SweB1 and the *N. caninum* reference isolate Nc-1 supports the identity of the Swedish isolate because ITS1 is considered to be conserved within species but to be more variable than individual rRNA genes between species (Hyde 1990).

In conclusion, we confirmed the identity of Nc-SweB1 as *N. caninum*, and by the methods applied we could not find any dissimilarity between this bovine isolate and the canine *N. caninum* isolate Nc-1. The findings not only confirm the identity of the Swedish isolate but also suggest that *Neospora*-like organisms isolated from cattle are indeed *N. caninum.*

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