Neospora caninum in cattle in Sweden

Isolation of the parasite and studies of its transmission

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Abstract

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Neospora caninum is a coccidian parasite with a two-host life cycle including a canine definitive host. In cattle the parasite is an important cause worldwide of abortion, stillbirth and calves born weak. This thesis, which deals with neosporosis in Swedish cattle, is based on five separate publications.

The brain of a stillborn calf, seropositive to *N. caninum* and born to a seropositive cow, was homogenised and cultured on Vero cells, where growth of Neospora-like tachyzoites was detected after 8 weeks. Electron microscopy, serology, immunoblot and polymerase chain reaction (PCR) revealed no dissimilarity between this new Swedish bovine isolate and previously published Neospora isolates, confirming that it was *N. caninum*. The isolate was designated Nc-SweB1.

In a herd of Swedish dairy cattle *N. caninum* antibodies were detected in 17 (29%) out of 58 animals. All seropositive cattle were descendants of two cows that were no longer alive. Evidence of congenital transmission of *N. caninum* and the apparent lack of other forms of transmission indicate the ability of *N. caninum* to be transmitted from dam to foetus for several generations.

Neospora caninum antibody levels of 13 cows were monitored during two pregnancies, and of five cows during one pregnancy. The mean antibody titres rose by 1.5-2.5 dilution steps to reach a plateau 4-5 months before parturition. This pattern was consistent in both pregnancies indicating a reactivation of the parasite rather than a reinfection at mid-gestation.

Four seronegative newborn calves were given cell culture-derived tachyzoites of *N. caninum* with their first feed of colostrum. Two calves fed by feeding bottle developed a lasting antibody response while the two calves dosed via stomach tubes remained seronegative throughout the study. This suggests that an oral route of *N. caninum* infection is possible in newborn calves.

A *N. caninum*-infected herd of 110 Swedish dairy cattle was studied over 5 years to monitor the transmission within the herd and the abortion rate. During the study the proportion of seropositive animals increased from 63% to 87%. A large part of this increase was attributed to vertical transmission, but 16 animals sero-converted apparently due to an additional horizontal route of infection, possibly including infective stages of the parasite spread by a definitive host. The abortion rate was more than twice as high as that of the average Swedish herd. An increase in abortion rate was the main detectable effect in this herd where a combination of vertical and horizontal transmission had led to *N. caninum* infection in almost all animals.

In this series of investigations the presence of *N. caninum* in Sweden is confirmed and it is shown how the parasite may be transmitted within herds. Processes in infected cows are demonstrated, and the potential clinical importance of *N. caninum* in Swedish dairy herds is illuminated.

Key words: cattle, protozoa, *Neospora caninum*, abortion, isolation of organism, experimental infection, transmission.

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Contents

Appendix

Papers I-V

The thesis is based on the following papers, which will be referred to by their Roman numerals:

I. Stenlund S, Björkman C, Holmdahl OJM, Kindahl H, Uggla A. Characterization of a Swedish bovine isolate of *Neospora caninum*. Parasitology Research 1997, 83, 214-219.

II. Björkman C, Johansson O, Stenlund S, Holmdahl OJM, Uggla A. *Neospora* species infection in a herd of dairy cattle. Journal of the American Veterinary Medical Association 1996, 208, 1441-1444.

III. Stenlund S, Kindahl H, Magnusson U, Uggla A, Björkman C. Serum antibody profile and reproductive performance during two consecutive pregnancies of cows naturally infected with *Neospora caninum*. Veterinary Parasitology 1999, 85, 227-234.

IV. Uggla A, Stenlund S, Holmdahl OJM, Jakubek E-B, Thebo P, Kindahl H, Björkman C. Oral *Neospora caninum* inoculation of neonatal calves. International Journal for Parasitology 1998, 28, 1467-1472.

V. Stenlund S, Kindahl H, Uggla A, Björkman C. A long-term study of *Neospora caninum* infection in a Swedish dairy herd. (Manuscript)

Abbreviations

BHV-1	bovine herpesvirus type 1
BVDV	bovine virus diarrhoea virus
CNS	central nervous system
ELISA	enzyme-linked immunosorbent assay
IFAT	indirect fluorescent antibody test
IgG_1	immunoglobulin G_1
N. caninum	*Neospora caninum*
Nc-SweB1	a Swedish *Neospora caninum* isolate of bovine origin
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
SHS	Svensk Mjölk (Swedish Dairy Association)
SLB	svensk låglandsboskap (Swedish Friesian breed)
SRB	svensk rödbrokig boskap (Swedish red and white breed)
SLU	Sveriges lantbruksuniversitet (Swedish University of Agricultural Sciences)
SVA	Statens veterinärmedicinska anstalt (Swedish National Veterinary Institute)

Introduction

Neospora caninum is a protozoan parasite, which was first identified as a cause of canine paralysis. A later association with bovine abortions made the parasite a concern for the farming community worldwide. During the one and a half decades since the parasite was recognised, large steps have been taken towards understanding its biology and pathology. In this thesis the focus is on *N. caninum* in Swedish cattle; the isolation of the organism, studies of its transmission and the kinetics of *N. caninum*-specific antibodies in pregnant infected cattle.

General Background

Neospora caninum

The parasite

Neospora caninum is a protozoan parasite of the family Sarcocystidae in the phylum Apicomplexa (Dubey, et al., 1988a), and bears a great similarity to *Toxoplasma gondii.* The parasite was first detected in 1984 in dogs with lameness (Bjerkås et al., 1984) and was for the first few years only connected with disease in dogs. Around 1990 its association with bovine abortion was unravelled (Thilsted and Dubey, 1989; Anderson et al., 1991; Barr et al., 1991). *Neospora caninum* has a worldwide distribution and has for the past 10 years been known as one of the most commonly diagnosed causes of bovine abortion.

Life cycle

Neospora caninum has a two-host life cycle with the dog as a definitive host (McAllister et al., 1998). The definitive host, that is the host in which the sexual reproduction of the parasite takes place, was unidentified for many years. A carnivore was suspected, but only in 1998 did researchers find oocysts in the faeces of dogs fed *N. caninum*-infected mice. The oocysts are sub-spherical, 10-11µm in diameter, and unsporulated at shedding. Oocysts sporulate within 3 days and then contain two sporocysts, each with four sporozoites (McAllister et al., 1998; Lindsay et al., 1999b). The oocysts shed in the experiment were few and one of the oocyst-shedding dogs did not sero-convert to *N. caninum* (McAllister et al., 1998). It is still not known whether the period of oocyst shedding can recur and, if so, how often (Dubey, 1999). Also, the survival conditions of the oocysts in the environment are not known (Dubey, 1999).

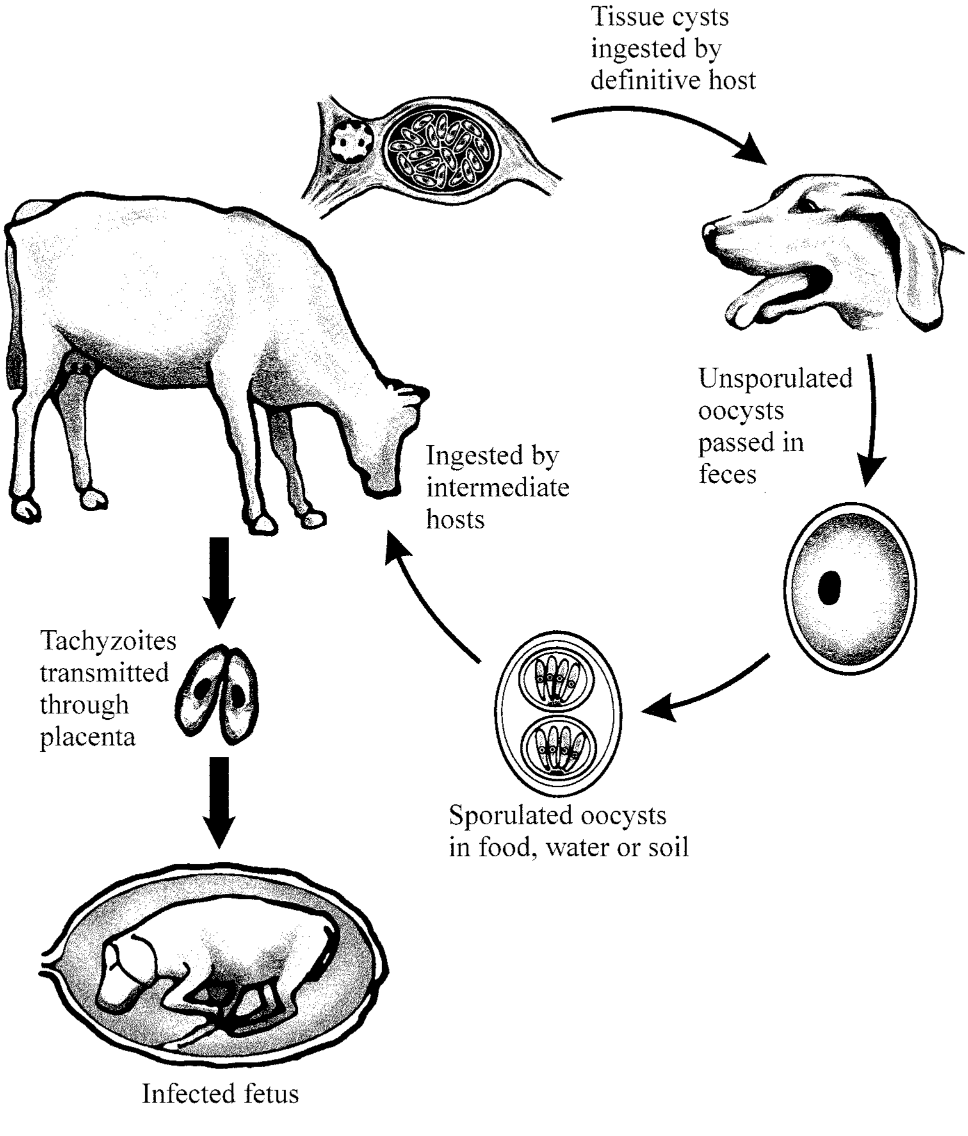
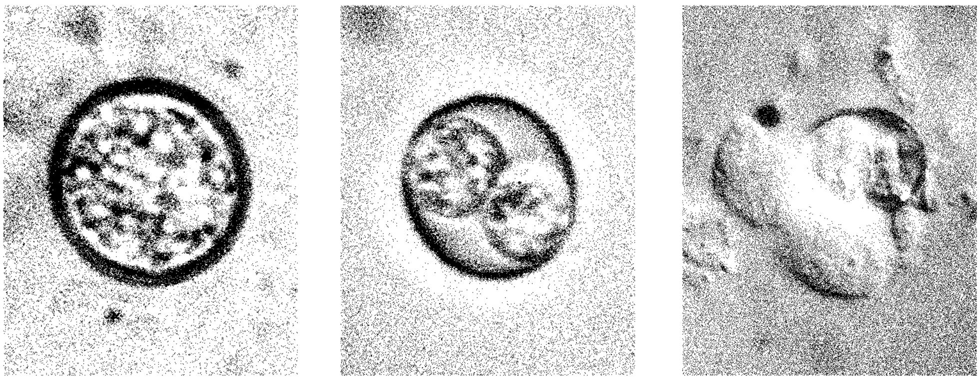


Figure 1. The life cycle of *Neospora caninum*. The dog is a definitive host of the parasite and can shed oocysts with its faeces. Oocysts can be ingested by intermediate hosts such as the cow. Tachyzoites may be transmitted from the dam to its foetus. This is the main form of transmission for the parasite. The dog may ingest tissue cysts via uncooked tissue from an intermediate host, thus completing the life cycle of the parasite. Modified from figure published in Veterinary Parasitology 84 (1999) (with kind permission of Dr. JP Dubey)

Several mammalian species can act as intermediate hosts for *N. caninum*, for example cattle, sheep, goats, deer, horses and probably, as indicated by serological evidence, water buffaloes, coyotes, red foxes and camels. In addition, a number of further species have been found possible to infect experimentally (reviewed by Dubey, 1999). As a natural infection, the parasite has, however, most often been found in cattle and dogs. Human exposure to the infection has been shown in one study (Tranas et al., 1999) but not in any of the studies that have been performed to investigate a possible zoonotic aspect of the disease (Petersen et al., 1999; Graham et al., 1999). Non-human primates infected experimentally have been found to transmit the infection to their offspring during pregnancy (Barr et al., 1994).

Neospora caninum, in the form of a tachyzoite, penetrates host cells by active invasion (Hemphill et al., 1996). The tachyzoites are 3-7×1-5 μm in size, and ovoid, lunate or globular in shape, and divide by binary fission. They can be localised in many different cells, but are most often found in neural cells (Bjerkås and Presthus, 1989; Speer and Dubey, 1989; Wouda et al., 1997b). The tachyzoites are situated in a parasitophorous vacuole within the host cell cytoplasm, and they have an apical complex consisting of two apical rings. They also have a conoid, up to 150 micronemes and 8-18 rhoptries, some extending posterior to the nucleus (Speer and Dubey, 1989; Lindsay et al., 1993). The fast growing tachyzoites are eliminated by the host's immune response which drives the development towards the slowly dividing encysted bradyzoite stage (Hemphill, 1999). The bradyzoites are slender (6-8×1-1.8 μm) and contain the same organelles as do the tachyzoites, but have fewer rhoptries (Jardine, 1996). Tissue cysts are found in neural tissues. They are round to oval, and usually measure 5-27×6-31 μm. The cyst walls are smooth and most commonly 0.2-2 μm thick (Bjerkås and Presthus, 1988; Jardine, 1996).



a b c

Figure 2. Oocysts of *Neospora caninum*: (a) unsporulated oocyst; (b) partially sporulated oocyst; (c) sporulated oocyst. (Photo: M McAllister)

The intestinal stages in the definitive host are a prerequisite to the parasite's sexual reproduction. *Neospora caninum* can, however, also be transmitted from mother to foetus during pregnancy. This was for some time its only recognised form of transmission (Paré et al., 1996).

Pathological lesions

Rapid intracellular multiplication of *N. caninum* tachyzoites causes cell death which can lead to grossly visible necrotic lesions within a few days (Lindsay et al., 1999a). Tachyzoites are mainly found in brain and neural tissues where they can destroy large numbers of neural cells, including those of cranial and spinal nerves (Barber et al., 1996; Lindsay et al., 1999a). A focus of mononuclear cell infiltration surrounding a central area of necrosis is the characteristic lesion of neosporosis in the CNS. Tachyzoites also affect the conductivity of the infected cells, and can produce severe neuromuscular disease in dogs, cattle and probably other hosts (Mayhew et al., 1991;Dubey and Lindsay, 1996). Tissue cysts of *N. caninum* are usually not associated with lesions (Wouda et al., 1997b). It is not known how long the cysts can persist in the central nervous system (CNS) but they have been known to remain viable in experimentally infected mice for at least 1 year (Lindsay et al., 1992).

In canine cases lesions are described mainly in the CNS and the peripheral nervous system, and secondarily in muscle and other tissues (Barber et al., 1996; Dubey and Lindsay, 1996). Aborted bovine foetuses are often autolysed or mummified and therefore difficult to examine. Gross lesions are rare but may be found as pale foci in skeletal muscles and in the heart, or as small pale to dark foci of necrosis in the brain (Dubey and Lindsay, 1996). In one study of bovine foetal neosporosis, histologic lesions in the brain, heart and liver were observed in 91% of the 80 cases (Wouda et al., 1997b). Degenerative and inflammatory lesions can also be found in skeletal muscle and other foetal tissues (reviewed by Dubey and Lindsay, 1996).

Immunity

Experimental *N. caninum* infection causes no symptoms in non-pregnant cattle, or in certain strains of mice, suggesting that the immune systems of these animals are able to control the infection. It has been shown that *N. caninum* elicits both humoural and cell-mediated immune responses in infected animals (Lundén et al., 1998; De Marez et al., 1999). Experimental infection of cattle with *N. caninum* has been shown to elicit an IgG antibody response within 3 weeks (Conrad et al., 1993b; Lundén et al., 1998). Presence of antibodies does probably not ensure control of the infection, which most likely largely depends on cell-mediated immune mechanisms (Khan et al., 1997; Lundén et al., 1998). Even though the details of the cell-mediated immune response are not yet known, a protective role of cytokines has been shown in mice. Some strains of mice have a natural resistance to *N. caninum* depending on interleukin 12 (IL-12) and interferon-γ (Khan et al., 1997), while treatment with the same cytokines renders sensitive strains more resistant (Baszler et al., 1999b).

Cultivation

In vitro cultivation of *N. caninum* has been achieved in many cell types. When the parasite was first isolated from a diseased dog in 1987 bovine monocytes and bovine cardiopulmonary arterial endothelial cells were used (Dubey et al., 1988b). The parasite has since then been grown in several well-established cell lines, amongst others Vero cells (Barber et al., 1993,1995; Dubey and Lindsay, 1996). The tachyzoite is the form of *N. caninum* which is generally seen in cell culture (Dubey et al., 1988b). Recent studies have shown that development of the bradyzoite form in cell culture can be provoked by extreme culturing conditions (Weiss et al., 1999).

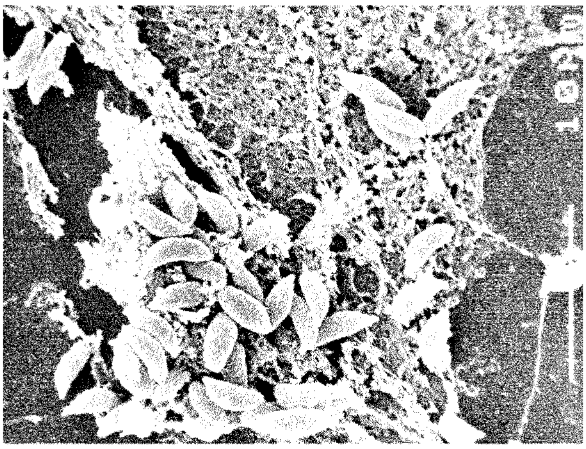


Figure 3. Tachyzoites of *Neospora caninum.* (Photo: B Koudela)

Organisms from cell cultures are infective for animals (Lindsay and Dubey, 1990) and tachyzoites have been passaged continuously for 8 years, with no loss of infectivity for mice (Dubey and Lindsay, 1996). They can also be cryopreserved and retain their infectivity (Dubey and Lindsay, 1996).

Neospora caninum *in dogs*

Most cases of canine neosporosis are diagnosed in young, congenitally infected pups. They develop hind limb paresis and ataxia which become progressively more severe (Dubey and Lindsay, 1993; Barber and Trees, 1996). Adult dogs may also suffer from neosporosis and clinical disease has been seen in a 15-year-old dog (Dubey et al., 1988a). Early antiprotozoal treatment can lead to recovery in approximately 50% of the cases (Barber and Trees, 1996). Bitches have been seen to transmit the parasite to their foetuses during pregnancy in successive pregnancies (Barber and Trees, 1998).

Bovine pregnancy and abortion

The average gestation time in cattle is 280-285 days, depending to some extent on breed (Peters and Ball, 1995). For the two main breeds in Sweden, the Swedish Friesian (SRB) and the Swedish red and white breed (SLB), the mean gestation time is 280 and 278.5 days, respectively (SHS Årsstatistik, 1988-1999).

Abortion is defined as an expulsion of the foetus between 42 days after conception and approximately 260 days of gestation. Before day 42, embryonic death generally leads to resorption, or expulsion with no clinical signs in the cow. Expulsion of the foetus after 260 days is regarded as an early parturition (Anonymous, 1972). The definition "stillborn" is used if the calf is dead at birth or dies within 24 hours thereafter.

The cause of abortion is often difficult to determine. Most abortions are considered due to non-infectious causes such as genetic disorders, teratogens, nutritional disorders, toxic plants, hormonal asynchrony, corticosteroids, physical insults, and endotoxins (Larson, 1996). Infectious agents such as viruses, bacteria, fungi, yeasts or protozoa can also lead to abortion (Barr and Anderson, 1993; Larson, 1996).

A specific diagnosis is obtained for only 23-46% of foetuses submitted to diagnostic laboratories. In most of these cases infectious diseases are diagnosed (Barr and Anderson, 1993). In a 10-year study of nearly 9000 aborted bovine foetuses in the USA, 33% of the abortions were diagnosed (Kirkbridge, 1992). An infectious cause was established for 30% and a non-infectious cause, for 2.5% of the abortions; 14% of abortions were due to a bacterial, 11% to a viral and 5% to a fungal infection. However, this study was performed prior to the development of appropriate methods of diagnosing *N. caninum* abortions.

The abortion rate in Swedish dairy herds is reported to be less than 1% (SHS Årsstatistik, 1988-1999). This figure is, however, probably an underestimation since not all abortions are reported to the Dairy Association. Internationally, approximately 2-5% of sporadic abortions are expected in typical dairy herds (Roberts, 1986).

Bovine virus diarrhoea virus (BVDV) has been considered the most important infectious abortive agent in Sweden. In 1993 at least 25% of all abortions were attributed to BVDV (personal communication, Rauni Niskanen and Stefan Alenius, SLU). The same year an eradication programme designed to eliminate the disease was initiated. After 7 years of eradication activity the incidence of disease has been drastically reduced, and abortions due to BVDV are now calculated to be considerably fewer than in the early 1990s (personal communication, Stefan Alenius, SLU)

Other abortifacients diagnosed in Swedish herds during the past 10-15 years, are *Listeria monocytogenes* and *Ureaplasma diversum*. However, *Brucella abortus*, *Campylobacter foetus*, *Leptospira hardjo*, *Tritrichomonas foetus*, and bovine herpesvirus type 1 (BHV-1), which are important abortive agents in many parts of the world, are not present in the Swedish bovine population (personal communication, Kerstin de Verdier Klingenberg, SVA).

Neospora caninum infection in cattle

Symptoms

Non-pregnant adult cattle that are infected with *N. caninum* do not show any signs of disease; pregnant cows, on the other hand, may abort their foetus. Abortions may occur irrespective of whether the infection in the cow is recent, chronic or congenital (Thurmond and Hietala, 1997a; Moen et al., 1998; Wouda et al., 1998). Furthermore, *N. caninum*-infected cows can abort in successive pregnancies (Anderson et al., 1995; Thurmond and Hietala, 1997a). *Neospora caninum*-associated abortions can occur during all stages of pregnancy and may include stillbirth at full term, although abortion at mid- to late gestation is the most common (Anderson et al., 1995; Otter, 1997).

Neospora caninum-infected calves are usually born healthy but persistently infected. These calves do not seem to be at any disadvantage. On the contrary, in one substantial study, infected calves experienced significantly less mortality during the pre-weaning period than did non-infected calves (Paré et al., 1996). Some of the congenitally infected calves are born alive but are diseased, or become ill within the first 2 months of life. The parasite affects the CNS, resulting in neurological signs. Hind limbs and/or forelimbs may be flexed or hyper-extended. The calves may be recumbent, and may exhibit ataxia and/or loss of conscious proprioception. Some infected calves are merely underweight. However, a small number of calves from infected cows are born uninfected.

Diagnosis

The presence of antibodies to *N. caninum* in serum indicates that the animal is, or has previously been, infected. Antibodies can be demonstrated by the indirect fluorescent antibody test (IFAT) (Dubey et al., 1988b), enzyme-linked imunosorbent assay (ELISA) (Paré et al., 1995, Björkman et al., 1997) or by the direct agglutination test (Packham et al., 1998; Romand et al., 1998). An IgG avidity ELISA (Björkman et al., 1999) can be used to discriminate between recent and chronic *N. caninum* infections in cattle. For a review on serological diagnosis see Björkman and Uggla (1999).

Figure 4. Tissue cyst of *Neospora caninum* in ovine brain. (Photo: K Gustafsson)

For a definitive diagnosis of neosporosis, a necropsy of the foetus or calf is necessary. Samples from the brain, heart and liver should be collected and examined for histopathologic changes (Dubey, 1999), and body fluids or serum examined for presence of antibodies (Björkman and Uggla, 1999). Since there are no pathognomonic gross or histopathologic lesions of neosporosis, the presence of *N. caninum* in the tissue must be identified for the diagnosis to be established. The organisms cannot be differentiated from other apicomplexan species by standard histologic stainings but they can be identified by immunohistochemistry (Lindsay and Dubey, 1989). In this method the parasite is selectively stained by using specific antibodies to *N. caninum*. *Neospora caninum* DNA can be detected by polymerase chain reaction (PCR). Several methods have been reported (Holmdahl and Mattsson, 1996; Gottstein et al., 1998; Ellis, 1998; Baszler et al., 1999b). However, there are usually very few *N. caninum* organisms present even in the CNS of infected animals. The sensitivity to detect parasites is therefore low (Dubey, 1999; Thurmond et al., 1999). The presence of *N. caninum* antibodies in body fluids or serum from a foetus or pre-colostral calf indicates infection. However, the absence of antibodies does not rule out neosporosis. The foetus may not have been immunocompetent at the time of infection, or else, the time between infection and abortion may have been too short for antibodies to develop (Wouda et al 1997a)

Epidemiology

Neosporosis in cattle has been reported from countries and regions worldwide. In some regions a large proportion of bovine abortions is considered to be caused by *N. caninum*. In California and The Netherlands the parasite has been reported to cause 20-43% and 17%, respectively, of all bovine abortions (Anderson et al., 1991, 1995; Wouda et al., 1997b).

In Sweden the prevalence of *N. caninum* in dairy cattle is estimated to be 2%. Antibodies to *N. caninum* have been found in sera from 7% of dairy cattle in herds with abortion problems (Björkman et al., 2000).

Neospora. caninum affects both dairy and beef cattle (Hoar et al., 1996; Waldner et al., 1998; 1999). The abortions caused by the parasite can be sporadic, clustered or epidemic (McAllister et al., 1996; Moen et al., 1998; Wouda et al., 1998) and can occur all year round. Cows with *N. caninum* antibodies are more likely to abort than *N. caninum* are seronegative cows (Thurmond and Hietala, 1997a; Moen et al., 1998; Wouda et al., 1998).

Transplacental transmission of *N. caninum* to the offspring has been documented to occur during consecutive pregnancies in the same cow. In cattle *N. caninum* is efficient in being transmitted vertically, even for several generations (II; Anderson et al., 1997; Schares et al., 1998). For many years this was in fact thought to be the only form of transmission for *N. caninum*. However, since there were reports of abortion storms associated with *N. caninum* infection, the presence of horizontal infection has been suspected (Yaeger et al., 1994; McAllister et al., 1996, 2000). Horizontal transmission appears to be necessary to introduce new infection into the herd (Thurmond et al., 1997; French et al., 1999).

The Present Study

Aims

The present study deals with various aspects of *N. caninum* infection in cattle in Sweden. The first objective was to confirm the presence of the parasite in Swedish cattle by isolation of the organism from the tissues of a Swedish bovine.

Further objectives were to describe different modes of transmission occurring in herds with naturally infected cattle, as well as to study the possibility of *N. caninum* to be transmitted galactogenically, and to describe the antibody kinetics during pregnancy in chronically infected cows.

Methodological considerations

Detailed descriptions of materials and methods used are given separately in each paper (I-V).

Isolation of Neospora caninum *(I, IV)*

During the course of the present work, several attempts were made to isolate *N. caninum* from aborted or stillborn calves as well as from canine pups with neurological symptoms. Attempts were also made to re-isolate the parasite from calves experimentally infected with the Swedish isolate of bovine origin (Nc-SweB1) using the same method as described in Paper I.

Animals and tissues

Most of the tissues used in the attempts to obtain isolates of *N. caninum* were from aborted calves submitted to the Pathology Department of the Swedish National Veterinary Institute (SVA) for post-mortem examination (I). Some of the cases were foetuses or calves born to dams known to be seropositive to *N. caninum*. The stillborn calf from which Nc-SweB1 was isolated had been born to a seropositive cow, and originated from a farm with a known high seroprevalence of *N. caninum*. To avoid contamination the brain was removed aseptically, immersed in phosphate-buffered saline (PBS) with antibiotics, and thereafter flamed before samples for cell culture and histology were removed.

Purification and cultivation

Nine attempts to isolate *N. caninum* from naturally infected animals were made before the successful isolation of the Nc-SweB1. *Neospora caninum* is believed to be sparsely distributed in the CNS (Wouda et al., 1997b), and no-one has yet shown a predilection site for the parasite. Therefore it was desirable to use as much of the brain as possible in each isolation attempt. The brain used in this study was homogenised by pestle and mortar and digested with trypsin in PBS. Percoll® (Amersham Pharmacia Biotech, Uppsala, Sweden) was then used to separate the fraction containing parasites from debris and fat, thus reducing the amount of material with which to inoculate the cell flasks. This method made it possible to use a large proportion of the brain for isolation.

In this study Vero cells (Barber et al. 1995), originating from monkey kidney, were used for the isolation attempts. Others have used bovine cell lines (Dubey et al., 1988b; Conrad et al., 1993a). A conceivable advantage of using a bovine cell line when attempting to isolate the organism from a calf is that the parasites would not have had to adapt to cells from a new species. However both bovine and non-bovine cell lines have been used in isolating *N. caninum* from cattle with equal success (Yamane et al., 1998; Fioretti et al., 2000)

Figure 5. Tachyzoites of *Neospora caninum*. (Photo: B Koudela)

The growth rate of the Vero cells was minimised in this study by using cell culture medium supplemented with a low level (2%) of foetal calf serum, only changing the medium twice weekly, and by sub-culturing the cells at 10- to 11-day intervals. This was a low level of sub-cultivation, which kept down the expansion rate of Vero cells but may have provided the organisms with a sub-optimal environment.

Epidemiological studies (II, III, V)

Animals and herds

Two herds that had experienced problems with abortions were included in the study. They were herd A in Paper II and herd B in papers III and V. Herd B had a *N. caninum* prevalence of 63%. The herd was included in a longitudinal study (V) with the assumption that the prevalence was too high for culling of seropositive animals, and therefore, that the population was likely to be kept as if no knowledge of the infection existed.

Mainly female animals were included in studies II and V. The males were included only as offspring, to add information on possible vertical transmission. They were sampled but remained in the herd for too short a period to add further information on sero-conversion. All animals above 4 months of age were sampled. Colostral antibodies were estimated to have been eliminated at approximately that age based on knowledge of BVDV antibodies (Palfi et al., 1993). It was later shown by Hietala and Thurmond (1999) that colostral antibodies decay by 128 days in *N. caninum* seronegative calves born to seropositive cows.

Collection of data

Data for studies II, III and V were collected from the farm records and from the Milk Recording Service of the Swedish Dairy Association. Data on abortions are not always reported to the Milk Recording Service. In the case of incomplete data the farm records were used.

Serological analyses

Serum samples were analysed by an ELISA employing a *N. caninum* iscom antigen (iscom ELISA) (Björkman et al., 1997). The method has a sensitivity of 100% and a specificity of 96% as compared to the IFAT as the indicator of true status. An avidity ELISA (Björkman et al., 1999) was used in Paper V to differentiate between recent and chronic infection.

Experimental infections (IV)

Inoculation

Viable parasites are a prerequisite to a successful experimental infection. To ensure their viability the organisms were maintained at 37°C, from the moment they were removed from the incubator until dosed to the calves. To confirm that the parasites were viable, a sample from the batch used to infect calves with were stained with Trypan blue and examined by light microscopy (Jakubek et al., 1997).

Statistics (II, III, V)

Statistical analyses were performed using Minitab® statistical software (Minitab Inc. State College, PA, USA). Student's *t*-test (V), χ^2 (II) and analysis of covariance using a general linear model (III), were used for data with a normal distribution. If the data were not normally distributed, the Mann-Whitney test (II) was used. Homogeneity of variances and outlying values (III) were tested for by plotting the residuals. *P*-values of <0.05 were considered significant.

Results and discussion

Isolation of Neospora caninum*, and experimental infection (I, IV)*

Isolation in cell culture

At the start of this study *N. caninum* had relatively recently been found to have a major role in bovine abortion. Many attempts had been made by different groups to isolate the parasite from cattle and the first successful isolations were made by Conrad et al. (1993a). After several attempts in Sweden, *N. caninum* was successfully isolated from a stillborn calf, and the isolate called Nc-SweB1 (I). Parasitic growth was detected in the cell culture flasks 56 days after the inoculation. This was longer than the 15-34 days observed by Conrad et al. (1993a). In later isolations, growth of bovine isolates was likewise detected after a comparatively long time (Yamane et al., 1997; Davison et al., 1999a; Magnino et al., 1999; Fioretti et al., 2000). Also, in one out of four isolations from calves using nude mice in an additional step between the calf and the cell culture, the parasites took as long as 35 days to become visible (Yamane et al., 1998).

One explanation for this long time period between the inoculation and detection of parasites in the cell culture flasks is the scarcity of parasites present in brains of infected animals (Wouda et al., 1997b). Autolysis is likely to further diminish the number of viable parasites. A low number of parasites inoculated in the original flasks are likely to take a long time to multiply sufficiently to become visible at ocular examination. No parasites were detected by immunohistochemistry in the present calf supporting the impression of a low number of parasites in the brain. Furthermore, it is not known what stage of the life cycle the parasites, being the origin of the Nc-SweB1 isolate, were in. If the calf had been infected recently they may have been tachyzoites; however, they may also have been bradyzoites from tissue cysts. Bradyzoites replicate slowly (Lindsay et al., 1999a; Hemphill 1999). Also, cell culture parameters such as host type may have had an influence on the time required for replication and growth. In this case, the host cells were from a different animal species than the original host. It is also conceivable that the technique of suppressing the host cell growth caused a sub-optimal environment for the parasites.

Once established, Nc-SweB1, exhibited a slow growth compared with other *N. caninum* isolates. Others have confirmed the slow growth of Nc-SweB1 (Schock et al., 1999) suggesting a specific characteristic of this isolate (Atkinson et al., 1999).

Avoiding bovine virus diarrhoea virus contamination

Foetal calf serum used in cell culture media may be contaminated with BVDV (Bolin et al., 1991). To be able to use the Nc-SweB1 isolate for experimental infections of pregnant cattle in a study of the pathogenesis of abortion, it was of utmost importance that the isolate was not contaminated with BVDV. Therefore the foetal calf serum used in the cell culture was confirmed free from BVDV following virus isolation performed at the SVA (Meyling, 1984). Further, frozen and sonicated Nc-SweB1 tachyzoites were analysed by the same method, and were found not to contain BVDV. A definitive confirmation that the isolate was BVDV-free was obtained by the experimental infection of 10 calves (unpublished results) originating from herds declared free from BVDV by the Swedish national eradication programme. Sera collected 3 months after inoculation were analysed by ELISA (Juntti et al., 1987) and were found not to contain antibodies to BVDV. Cattle are very susceptible to BVDV and if the isolate had contained even a small number of the virus they would have been expected to sero-convert within 3-4 weeks, and to have reached a maximum antibody level within 3 months (Brownlie et al., 1987). The aspects of presence of BVDV in conjunction with experimental infections of *N. caninum* in cattle have not been addressed elsewhere.

Characterisation

A comparison of the ultra-structure of Nc-SweB1 with that of the canine isolates Nc-1 and Nc-Liv showed great similarity between the isolates. Also, a study of antigenic qualities by Western blot and IFAT revealed similarities with *N. caninum* isolates and clear differences to parasites of related Apicomplexan species. The alignment of the 16S-like rRNA and ITS1 showed complete homology between Nc-SweB1 and the two canine isolates, and a large number of sequence differences between Nc-SweB1 and *T. gondii*. The characterisation of Nc-SweB1 confirmed the identity of the isolate as being *N. caninum*, and also suggested that *N. caninum* in cattle and *N. caninum* in dogs belong to the same species (Holmdahl et al., 1997). Recently *Neospora*-like parasites were isolated from a horse in the USA and were proposed to constitute a new species, *N. hughesi* (Marsh et al., 1998). There were several nucleotide base differences between the isolate and *N. caninum*. Also, its tissue cyst and bradyzoite form had a different ultra-structure to that of *N. caninum* (Marsh et al., 1998).

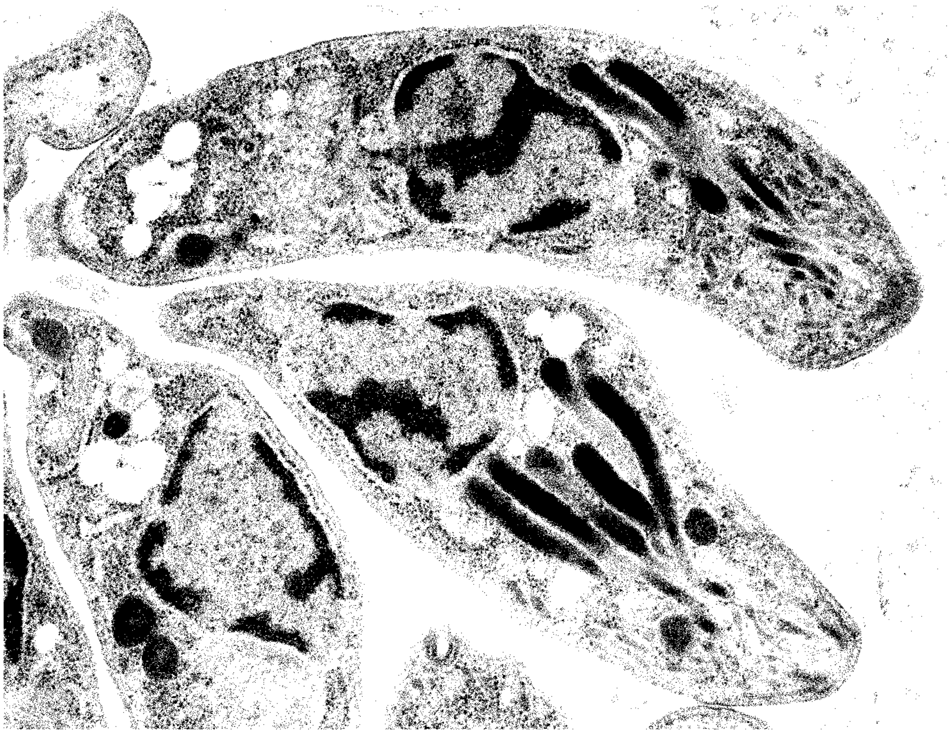


Figure 6. Tachyzoites of *Neospora caninum* (Nc-SweB1). (Photo: T Nikkilä)

Experimental infection

In the present study the Nc-SweB1 isolate was used to experimentally infect calves. In Paper IV neonatal calves were dosed orally and sero-converted after 4-5 weeks. The presence of *N. caninum* in their brains was demonstrated by PCR. Animals inoculated intramuscularly at 3-months' age sero-converted 3 weeks after inoculation (unpublished study). The doses used in the two experiments were of approximately the same size, but it is likely that most of the oral dose was destroyed in the digestive system and only a few organisms actually penetrated the mucosa and entered the host. This may have influenced the time required for sero-conversion.

One of the main aims of isolating *N. caninum* from a Swedish bovine was to confirm that the parasite is present in Swedish cattle after its presence had previously only been demonstrated by immunohistochemistry (Holmdahl et al., 1995). The Nc-SweB1 isolate was the first Swedish *N. caninum* isolate obtained and the first bovine *N. caninum* isolate from outside the USA; today it is maintained at SVA by serial passages in cell culture. The isolate has repeatedly been found to survive cryopreservation and thawing.

Transmission of Neospora caninum *(II, III, IV, V)*

Herds

Transmission of *N. caninum* was studied in two herds (A and B) with a high prevalence of animals seropositive to *N. caninum*. In herd A (II) the animals were tested once only but were studied retrospectively regarding kinship, abortions and reproduction problems. In this herd, 29% of the animals had antibodies to *N. caninum*. All seropositive animals were descendants of two cows that had been brought into the herd 13 years previously. All the seropositive female cattle (n=9) with mothers present in the herd at sampling, had seropositive mothers. This strongly indicates that *N. caninum* in this herd spread via vertical transmission only. In other words, the parasite was transmitted from cows to their offspring. Other studies have since confirmed that vertical infection is the main form of transmission in dairy herds (Paré et al., 1996; Schares et al., 1998; Davison et al., 1999c)

In herd B (V) a longitudinal study was performed. Here, as many as 63% of the animals had antibodies to *N. caninum* at the initial sampling. A look at their family trees as far back as 1940 established that the seropositive animals were not all related to each other. Therefore, it was not possible to trace likely ancestors of the present infected animals. The most striking difference between herd A and herd B was that most of the animals from herd B that were seropositive at the start of the study had seronegative mothers. Also, many young animals and few older animals had antibodies to *N. caninum*. This suggests that horizontal transmission had occurred recently in this herd. Low IgG avidity activities at the start of the study as compared with 5 years later also suggested recent infection in more animals at the beginning of the study than 5 years later.

During the course of the 5-year study the number of seropositive animals in herd B increased. This increase would be expected since no efforts were being made to selectively cull seropositive animals, and considering the shown efficiency of vertical transmission of *N. caninum* (II; Schares et al., 1998; Davison et al., 1999c; Thurmond et al., 1999). However, in addition there was an ongoing horizontal spread during the study period, reflected by the sero-conversion of 16 animals, eight of which sero-converted during the last year of the study, indicating that this transmission had not yet been controlled. Horizontal transmission, albeit at a low level, is considered to be present in many herds (Thurmond et al., 1997; Wouda et al., 1998; Davison et al., 1999c). Continuously high antibody levels to *N. caninum* over several years have been suggested to indicate repeated exposure to the infection (Hietala and Thurmond, 1999). If this is true, the high *N. caninum* ELISA absorbance values over the years in eight animals, and the constancy throughout of the mean of all animals would indicate that the infection was active over the years in herd B.

The situation regarding within-herd transmission of *N. caninum* in herds A and B appears to have been entirely dissimilar. In herd A, transmission seems to have been strictly vertical. The infection appears to have been present in the herd since it was established, that is, for 13 years, and then to have been propagated vertically only. However, some seropositive cows gave birth to seronegative offspring, causing natural elimination of the infection in some branches of the family tree. Studies elsewhere have shown that vertical transmission of *N. caninum* is very efficient, but 5-7% of the calves of seropositive dams can be expected to be non-infected at birth (Schares et al. 1998; Davison et al., 1999c). In herd B it was most likely that, although *N. caninum* may have been present for a number of years, the infection was spread both vertically and horizontally during the 5 years of the study. The horizontal spread appeared to have started, or at least been more extensive than previously, during the year before the study was initiated and to have caused an increase in the herd's abortion rate during the following year. In other studies, point source exposure to *N. caninum* has been suggested to precede outbreaks of bovine abortion (McAllister et al., 1996). In view of the possibilities of horizontal transmission it may be noted that on farm A, besides common native wild animals, a cat, but no dog, was present. In herd B, however, there were two dogs on the farm and there was an abundance of foxes and some badgers in the area. The second dog was introduced on farm B in the spring of 1998, which coincides with a notable number of sero-conversions between 1998 and 1999. Also, the first dog was introduced in early 1994, the year during which *N. caninum* appears to have been spread horizontally to a large number of animals. Before 1994 there had been no dogs on the farm. These two dogs were seronegative to *N. caninum*, but while it has been shown that the dog is a definitive host for *N. caninum* and thus able to shed oocysts, all infected dogs are not seropositive (McAllister et al., 1998). The young dogs in the current study were often in the cow shed during winter and had access to the grazing grounds during summer. Therefore it is not inconceivable that they were infected with *N. caninum* at some stage and spread the infection to a number of cattle during the period during which they shed oocysts. Furthermore, no definitive host has yet been identified among wild animals. *Neospora caninum* antibodies have been demonstrated in some species related to the dog, such as coyotes, foxes and dingoes (Lindsay et al., 1996; Buxton et al., 1997; Barber et al., 1997) but attempts to experimentally induce shedding of oocysts from them have failed (Lindsay et al., 1996; Dubey et al., 1996; McAllister et al., 1999). Also, it was not possible to link foxes to a high level of *Neospora* abortions on a dairy farm (Simpson et al., 1997), whereas sero-epidemiological studies have shown a relationship between *N. caninum* infections in dogs and cattle Wouda et al., 1999). In addition, farm dogs have been found to be a risk factor for *N. caninum* associated abortions in cattle (Paré et al., 1998; Bartels et al., 1999).

Pregnant cows

Serum antibody profiles during two pregnancies were studied in a group of seropositive cows (III) in herd B. The mean antibody titre level rose during pregnancy to reach a plateau 4-5 months before parturition, and decreased as of 2 months before parturition. This pattern was seen in both the first and the second pregnancy. Oestruses were not synchronised and consequently, the parturitions were not in synchrony. Therefore, this constant pattern of increase of *N. caninum* antibody titres in several pregnancies suggests reactivation of a latent infection in the cows, rather than suggesting re-infection. Re-infection would be expected to cause a rise in antibody levels at different times in different animals and different pregnancies. For re-infection to give a constant pattern such as seen in this study, the cow would have to be particularly receptive to infection at a certain stage of pregnancy and in one pregnancy after another which does not appear likely. Also, continuous exposure to feed or water contaminated with *N. caninum* oocysts would be necessary.

Reactivation has also been suggested by other researchers as an explanation for transmission of the parasite during several successive pregnancies, and for repeated abortions to occur in seropositive animals (Conrad et al., 1993b; Barr et al., 1997; Wouda et al., 1999). One possible explanation for the high antibody titres observed during pregnancy is that an increase in oestrogen levels may compromise the immune response to intracellular micro-organisms (Styrt and Sugarman, 1991), leading to a decreased control of *N. caninum* encysted in the dam's tissues.

The highest antibody titres in the cows studied in Paper III were recorded in three out of the five pregnancies that ended in abortion, and in one pregnancy that followed immediately after an abortion. An increase in antibody levels during pregnancy may reflect a *N. caninum* infection in the foetus. It can be assumed that the foetal replication of *N. caninum* was higher in the pregnancies that ended in abortion than in pregnancies with a normal course. However, the exact pathogenesis of a *N. caninum* abortion is not fully understood. Whether the parasite primarily causes damage to vital parts of the foetus or primarily to the placenta, thus causing the foetus to die, is not known. These results partly contradict those of Pare et al (1997), who report that high antibody titres during late pregnancy, rather predicted that abortion was not likely to occur. On the other hand, they predicted that the calf was likely to be born infected.

Newborn calves

Four newborn calves were dosed orally with Nc-SweB1 tachyzoites (IV). The purpose was to investigate whether galactogenic transmission is a possible route of infection during the neonatal period. Galactogenic transmission of *N. caninum* in mice has previously been suggested. One out of seven female mice, inoculated with *N. caninum* 5-15 days post-partum, transmitted the parasite to one of her five pups (Cole et al., 1995). The present study shows that oral *N. caninum* infection of newborn calves is possible with the first meal of colostrum. Two of the calves sero-converted within 3 weeks after inoculation and remained seropositive at the same antibody level for at least 2-3 months, indicating an active infection and not merely immunisation. After culling, *N. caninum* DNA was also demonstrated in the brains of these calves by PCR. However, only the two calves dosed via an infant feeding bottle sero-converted. The calves dosed via a gastric feeding tube into the rumen did not sero-convert during the 4 months of the study. Although the number of calves used in the experiment was too small to draw any definitive conclusions, the results suggest that tachyzoites of *N. caninum* may be able to enter the host by penetrating the mucosa of the oral cavity, the oesophagus, the abomasum, or the small intestine. Tachyzoites of *N. caninum* have been shown experimentally not to tolerate hydrochloric acid and pepsin (Lindsay and Dubey, 1990). In the newborn, however, the proteolytic activity in the abomasum is low, and the trypsin activity in the small intestine is counteracted by trypsin inhibitors present in the colostrum (Tizard, 1996). The possibility of transmission of *N. caninum* via milk is of interest for our understanding of the epidemiology of the parasite. However, the presence of *N. caninum* in bovine milk has not yet been shown in field situations. In an ongoing study in Sweden, no presence of *N. caninum* has thus far been demonstrated by PCR in bovine colostrum from seropositive cows (Per Thebo, SVA, personal communication).

Significance of Neospora caninum *in Swedish dairy herds (II, IV, V)*

Thorough studies of two Swedish dairy herds (II, V) were performed to investigate forms of transmission of *N. caninum* and to study the consequences of the infection on herd health and reproduction.

Abortions

The main consequence of *N. caninum* infection for the herds in this study was abortions. In herd A (II) the abortion rate for the 3 years preceding the blood sampling was 6% (data not shown). In herd B (V) the mean abortion rate was 2% before the start of the investigation and 9% during the 5 years of the study. The general abortion rate in Swedish dairy herds is reported to be less than 1% (SHS Årsstatistik, 1988-1999). This figure is, however, probably an underestimation since not all abortions are reported by farmers, insemination technicians and veterinarians. Even in view of an expected incidence of 2-5% abortions in typical dairy herds internationally (Roberts, 1986), the abortion rate in these two herds with a high prevalence of *N. caninum* infection was high. The average abortion rate of the *N. caninum* seropositive cows in herd B was 14% which is considerably higher than the Swedish average.

Three of four abortions in herd A occurred in seropositive cattle, whereas in herd B all the abortions (n=20) during the 5 years of observation occurred in seropositive cattle. Together, these figures show that in these herds the *N. caninum* seropositive cows were at a considerably higher risk of aborting their foetuses than were seronegative cows. This increased risk of abortion for *N. caninum* seropositive cows has also been shown in several studies from other countries (Thurmond and Hietala, 1997a; Thurmond et al., 1997; Paré et al., 1997; Wouda et al., 1998; Moen et al., 1998; Davison et al., 1999b).

In addition to abortion, *N. caninum* is also believed to cause stillbirth (Anderson et al., 1995; Graham et al., 1996) and neonatal mortality in cattle (Dubey et al., 1989). In fact, three of the available bovine isolates of *N. caninum* originate from stillborn calves (I, Yamane et al., 1998; Davison et al., 1999a). However, in herd B there was no difference in the number of stillbirths occurring between the years of the study and the years preceding the first visit to the farm.

Seropositive animals, and therefore, animals at increased risk of abortion, have accumulated in the two studied herds, but for different reasons. In herd A, the two ancestral cows thought to have introduced the infection, and their progeny, more frequently female, were kept in the herd because of their strong and healthy disposition. As this was a retrospective study the owners were not aware of the presence of *N. caninum* in the herd. In herd B the infection was known during the course of the prospective study, and the subsequent accumulation of infected animals was due rather to the original high concentration of seropositive animals, making it economically unrealistic to cull this category.

Fertility

Besides abortion and stillbirth, it is likely that *N. caninum* causes embryonic death and very early foetal abortion which may result in an increased number of inseminations per pregnancy or in prolonged intervals between inseminations (Larsson et al., 1994; Barker et al., 1998). However, in both herds described here the fertility was good and there were no certain signs of embryonic death. In herd A (II) the mean number of inseminations per pregnancy during the 13 years since the establishment of the herd was 1.55, which was lower than the mean for the region, of 1.70. In addition, no significant difference was seen between the number of inseminations per confirmed pregnancy in the group of cows presumed to have been infected with *N. caninum*, and the rest of the animals. Even though the average number of inseminations per confirmed pregnancy and year in herd B

(V), was lower during the 5 years preceding the start of the investigation (1.51) than during the 5 years of the study (1.70), this increase was too low and the number of animals too small for final conclusions to be drawn. An increased number of inseminations per confirmed pregnancy may also be caused by a number of other factors, such as missed heat detection and technical errors during insemination. Thus far no detailed studies have been published of early pregnancy loss in association with *N. caninum* infection (Trees et al., 1999).

Calf health

In the studies included in this work there was no evidence that *N. caninum* infection should have had any major effects on calf health. The inoculation of newborn calves (IV) resulted in very mild symptoms in the two infected calves. Also, in the two herds with naturally *N. caninum*-infected animals (II, V) the frequency of recorded calf disease was low during the periods studied. These findings concur with those of a large study in which congenital infection was found not to have a detrimental effect on calf health (Paré et al., 1996). In Paré et al. the congenitally *N. caninum*-infected calves survived the first 3 months of life better than did non-infected calves.

Economic consequences

Neospora caninum infection in itself may have a negative effect on milk production. This effect may also be observed as a consequence of abortion. In herd B (V) the mean annual milk production per cow fell from 8488 to 7543 l, between 1995 and 1997, after which it stabilised at a lower level. During the same period the regional mean increased from 7613 to 7762 l per cow. Thus, in contrast to the region where the mean production increased, the production in herd B decreased considerably. This may be explained by a change in feed since the farm entered an ecological animal production programme in 1995 and 1996. However, part of the decrease could be due to the *N. caninum* infection. In a study including only first-lactation dairy cows a significant decrease in milk production, of 2.5 lb/day, for seropositive compared with seronegative animals has been reported (Thurmond et al., 1997b). In addition, the abortion rate in herd B was high as compared with the Swedish average. The value of the calf itself lies mainly in its gender and whether or not it was born to a healthy and productive cow. The effect of abortion on milk production is complex and depends largely on the stage of gestation, and on whether or not the cow has been dried off after the previous lactation. Adverse economic effects of neosporosis may also be due to stillbirth, increased time to conception, increased culling or reduced value of breeding stock as reviewed and evaluated by Trees et al. (1999). Such parameters could not be evaluated with any certainty in the small sized herds studied here.

Concluding remarks

- This work concludes that *N. caninum* is present in the Swedish cattle population, causing abortion and stillbirth. This was shown by serology, immunohistochemistry, PCR and finally by isolating the organism from a stillborn Swedish calf.

- In a dairy herd the parasite may be spread entirely by vertical transmission, or by a combination of vertical and horizontal transmission. It can be assumed that one or more definitive hosts for the parasite are involved in the horizontal transmission in such herds.

- In herds where vertical transmission is the main route of infection a straightforward strategy to eliminate the infection is to avoid using infected heifers for breeding. Therefore, information to the farmers, availability of reliable diagnostics and the possibility of screening herds are important.

- The presence of *N. caninum* in a herd causes problems such as an increase in the abortion rate, resulting in economic losses. This is reason enough to limit the further spread of infection, and, ideally, to eradicate the parasite in Swedish bovines.

- The work indicates previously unknown forms of transmission of the parasite, by showing that it can be orally transmitted to a calf via colostrum. It is likely, however, that galactogenic transmission does not commonly occur.

- This work finally illuminates processes in the *N. caninum*-infected pregnant cow. The consistent manner in which the antibodies fluctuate during gestation indicates a reactivation rather than a re-infection of the parasite at mid-pregnancy.

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