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Serological evidence for naturally occurring transmission of *Neospora caninum* among foxes (*Vulpes vulpes*)

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Abstract

The study describes the time course of the *Neospora caninum*-specific antibody response in experimentally infected foxes, in naturally *N. caninum*-seropositive vixens and their litters. An immunofluorescence test, a tachyzoite surface antigen based ELISA and an immunoblot assay were established for this purpose. The immunoblot patterns of naturally seropositive and experimentally infected foxes revealed a high degree of similarity and resembled those reported for other intermediate host species. Reactions against immunodominant antigens of *Mr* 56, 68 and >94 kDa were observed which could be linked with a period of 14 days–2 months post experimental infection with tachyzoites. Cubs born by naturally seropositive vixens were found to be persistently or transiently seropositive, in the latter case, specific antibodies were detected only up to 44 days after birth. These antibodies may thus be of maternal origin. Differences between the immunoblot patterns of persistently positive cubs, those of their mothers and of transiently positive cubs, in particular the differential response to antigens of *Mr* 56 and 68 kDa, prove that cubs with persistent antibodies had actively mounted an antibody response. This result provides the first evidence for the postnatal or vertical transmission of *N. caninum* among naturally seropositive foxes.

Keywords: *Neospora caninum*; *Vulpes vulpes*; Red fox; Vertical transmission; Postnatal transmission; Experimental infection

1. Introduction

Neospora caninum is a frequently diagnosed cause of bovine abortion world-wide (Dubey, 1999). Vertical transmission or propagation seems to be important in the spread of *N. caninum* in cattle, but also in other host species including dogs, horses, domestic and wild or captive ruminants (Dubey, 1999). There is evidence that in addition to transplacental infection, postnatal transmission may play an important role in the epidemiology of bovine neosporosis (McAllister et al., 1996; Thurmond et al., 1997; Mainar-Jaime et al., 1999). Calves were postnatally infected by the experimental ingestion of colostrum containing *N. caninum* tachyzoites (Uggla et al., 1998). Another, possibly more important, source for postnatal infection might be oocysts shed by definitive hosts (De Marez et al., 1999). Experimental studies have shown that dogs can be definitive hosts of *N. caninum* and that one possible route of dog infection is the ingestion of tissues of infected intermediate hosts (McAllister et al., 1998; Lindsay et al., 1999). There is an ongoing discussion as to whether other canids, e.g. the fox, can also serve as definitive hosts for *N. caninum* (McGuire et al., 1999; Dubey et al., 1999; Wouda et al., 2000). The excretion of *Hammondia heydorni*-like oocysts, i.e. oocysts similar to those of *N. caninum*, by foxes has been reported (Ashford, 1977; Entzeroth et al., 1978; Gjerde, 1983). Antibodies against *N. caninum* have been found in field sera from red foxes, indicating that the fox is at least an intermediate host for *N. caninum* (Barber et al., 1997; Buxton et al., 1997; Simpson et al., 1997). However, these results have not been confirmed by experimental infection and the assumed natural infections have only been demonstrated by indirect techniques, i.e. antibody detection.

The aim of this study was thus to characterise the antibody response of red foxes to experimental infection with *N. caninum*, to compare this response with that observed in naturally seropositive red foxes using different serological methods and to search for serological evidence for vertical or postnatal transmission of *N. caninum* in naturally seropositive foxes.

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2. Materials and methods

2.1. Parasites

The NC-1 isolate of *N. caninum* (Dubey et al., 1988) was maintained as previously described (Schares et al., 1998). Tachyzoites were used immediately for IFAT and experimental infection or frozen at −80°C until used for immunoblot (IB) or ELISA.

2.2. Sera

To characterise the *N. caninum*-specific humoral immune response of foxes in ELISA, IFAT, and IB, sera from five farm foxes kept in cages at the Federal Research Centre for Virus Diseases of Animals (FRCV) were used.

At the age of 4 months, three of the foxes (one male and two females) were experimentally infected with *N. caninum* (NC-1 isolate; 1.0×10^7 tachyzoites given i.v.). Two foxes (male and female) were used as controls and kept in neighbouring cages. All foxes were fed pelleted dog food during the entire experimental period. Foxes were initially bled in weekly, later in 2-weekly, and finally in monthly intervals.

In May 2000, during a serological screening of foxes on a fur farm, a positive reaction against *N. caninum* was found in a 1-year-old vixen (T2535). This fox, her siblings and her mother had been used for investigating the role of maternal antibodies in foxes vaccinated against rabies (Müller et al., 2001). A collection of sera of members of this fox family had been stored at −20°C in the serum bank of the FRCV. To study vertical transmission in serologically positive, putatively naturally infected foxes, sera from four litters of this family were analysed. Two litters had been born by the same vixen (T5320) in 1998 and 1999. Two other litters had been born by two daughters of vixen T5320 in 2000. One of the daughters (T2535) had given birth to five cubs. Four of the cubs were killed by their mother a few days after birth. The other daughter (T4376) gave birth to three cubs. All surviving cubs were bled at least twice (about 1–1.5 and 2–2.5 months after birth). For some cubs, sera from further sampling dates were available (for details, see Fig. 3).

To estimate the *N. caninum* seroprevalence in spring 1998, 1999 and 2000 among the vixens of the fur farm, 41 (1998), 41 (1999), and 40 (2000) sera stored in the serum bank at the FRCV were tested.

2.3. IFAT and immunoblot

The IFAT was performed as described (Schares et al., 1998, 1999a) using an anti-dog IgG (whole molecule) FITC conjugate (Sigma) at a dilution of 1:50. Serum dilutions started at 1:25. A titre of ≥1:50 was deemed as positive.

IB was performed as described (Schares et al., 1998, 1999a) using fox sera at a dilution of 1:10 for screening (i.e. estimation of serum prevalence on the fur farm) and 1:100 for comparing IB patterns among seropositive foxes. Anti-dog IgG(H + L) peroxidase conjugate (Dianova) was used at a dilution of 1:500. The reactivities of sera with immunodominant tachyzoite antigens of *Mr* 17, 29, 30, 33, and 37 kDa (Barta and Dubey, 1992; Bjerkas et al., 1994; Paré et al., 1995; Atkinson et al., 2000) were recorded. The recognition of at least two of these immunodominant antigens was regarded as positive (Schares et al., 1999a; Atkinson et al., 2000).

2.4. ELISA

Affinity purified p38 antigen (Schares et al., 1999b) was used to sensitise ELISA plates (Nunc-Immuno (Polysorb)). The ELISA was performed essentially as described (Schares et al., 2000) at a serum dilution of 1:100. As a conjugate, monoclonal anti-dog IgG biotin (Sigma, Clone DG-22) was used at a dilution of 1:2000. A pool of sera collected 3–7 months after experimental *N. caninum* infection of the three foxes served as a positive control. A similar pool of sera from the control foxes was used as a negative control. For each sample, index values were determined as described (Schares et al., 1999a). The ELISA cut-off value of 0.052 used in the present study was based on the index values obtained for sera of one of the non-infected control foxes, Fox 1 (mean + 6 × SD). ELISA results between 0.031 (mean + 3 × SD) and 0.052 were regarded as inconclusive.

2.5. Computing and statistics

Confidence intervals for the estimated seroprevalences among vixens of the fur farm were calculated using the statistical package R1.00.1 (Ihaka and Gentleman, 1996) assuming a hypergeometric distribution.

3. Results

3.1. Antibody reactions in experimentally infected foxes

In the IB, the first antibody reactions in the experimentally infected foxes were detected 8 days p.i. (Fig. 1). On day 10 p.i., the sera of all foxes recognised immunodominant antigens of *Mr* 29, 30, 33 and 37 kDa. The first reactions against an immunodominant antigen of *Mr* 17 kDa were observed on days 22–31 p.i. Reactions against immunodominant antigens of 17, 29, 30, 33 and 37 kDa became weaker from day 62 p.i. onwards. Strong but transient reactions were observed against antigens of *Mr* 56, 68 and >94 kDa from day 13 to 22 p.i. onwards. Strong reactions against these three antigens remained visible until 31–62 days p.i. In the uninfected controls, no reactions were visible in IB analysis.

The IFAT titres started to rise 6–8 days after experimental infection (Fig. 2a). Maximum titres were reached between days 15 and 27 p.i.

In the ELISA, antibody reactions against the *N. caninum* p38 surface antigen became positive at day 8 or 10 p.i.,

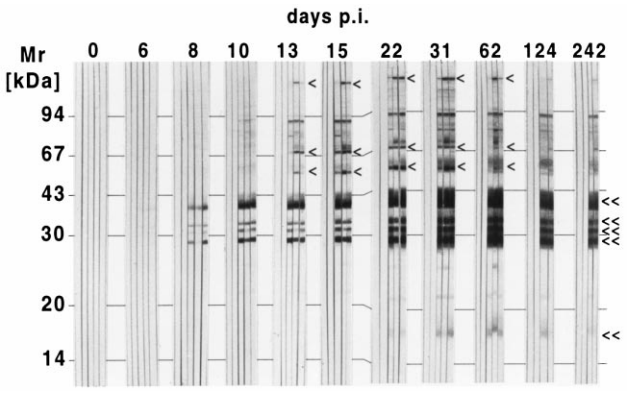

Fig. 1. Immunoblot results on non-reduced *Neospora caninum* tachyzoite antigens probed with fox sera. The results for different sampling dates for foxes 1 and 2 (uninfected controls) are shown in lanes 1 and 2, respectively, and for foxes 3–5 (experimentally infected animals) in lanes 3–5. Note: Fox 4 died 120 days post infection, therefore, in the cases of sampling dates 124 and 242, the results for fox 5 are displayed in lane 4. Reactions with immunodominant antigens of *Mr* 17, 29, 30, 33 and 37 kDa are indicated (<<). Transient strong reactions of foxes 3–5 with antigens of *Mr* 56, 68 and >94 kDa are also marked (<).

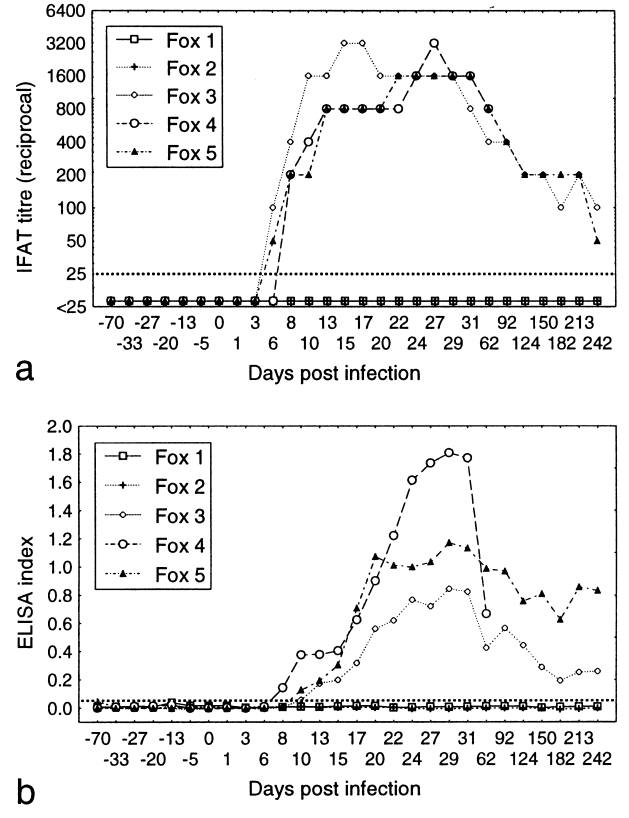

Fig. 2. (a) IFAT; and (b), ELISA results for sera from experimentally infected (foxes 3–5) and uninfected foxes (foxes 1 and 2). The cut-off used in the respective test is indicated (···). Fox 4 died 120 days post infection.

followed by a strong increase in antibody levels (Fig. 2b). Maximum ELISA indices were observed 29 days p.i.

3.2. Estimated prevalence of N. caninum*-specific antibody responses on the fur farm*

To estimate the N. caninum seroprevalence, 41 of 80 (1998), 41 of 80 (1999), and 40 of 106 (2000) vixens kept on the farm were screened by IB. Only one vixen/year showed a *N. caninum*-specific antibody response (Vixen T5320, T2535). Thus, the estimated seroprevalence for 1998 and 1999 was 2.4 (1.3–8.8%; 95% CI) and 2.5% (0.9–10.4%; 95% CI) for 2000.

3.3. N. caninum*-specific antibody response in the progeny of a naturally* N. caninum*-seropositive farm fox*

The time course of the *N. caninum*-specific antibody response was analysed in the progeny of a naturally *N. caninum*-seropositive vixen (T5320, Fig. 3). This animal showed antibody reactions in IB, IFAT and ELISA throughout the observation period (Fig. 3).

In 1998, vixen T5320 gave birth to a litter consisting of three cubs (O591/1-3). When 44 days old, these cubs tested *N. caninum*-positive in the IB, while only two of them were positive in the ELISA and IFAT. When the cubs were 71 days old, the cub which had initially been negative in IFAT and ELISA was negative also in the IB. At both sampling dates, one of the cubs showed a very high IFAT titre (1:6400), higher than the titre in a sample taken from its mother at the same time and higher than any other naturally or experimentally seropositive fox in this study.

In 1999, vixen T5320 gave birth to a second litter. Initially, on day 41 after birth, all cubs were positive in the IB. Two of them remained positive, while the other cubs turned negative in this assay later on (Fig. 3). One of the IB-positive cubs was also positive in the IFAT and ELISA until the end of the observation period. The second cub became positive in IFAT and ELISA at the second bleeding date, but the ELISA results turned inconclusive from the third bleeding onwards.

Two of the cubs born in 1999, T2535 and T4376, gave birth to two litters. All cubs born by vixen T4376 were seronegative. Vixen T2535 killed all her cubs except one. This cub was IB-positive on the first and the second sampling date, but then turned negative. In IFAT and ELISA, no specific antibody reactions could be detected.

3.4. IB characteristics of transient and persistent seropositive cubs born by seropositive foxes

The antibody response as detected by IB in cubs with a persistent *N. caninum*-specific antibody response (i.e. a positive result after day 44 after birth in either IB, IFAT or ELISA) was compared: (i), with the response of cubs with a transient antibody response (i.e. no positive serological results after day 44 postpartum); and (ii), with the antibody

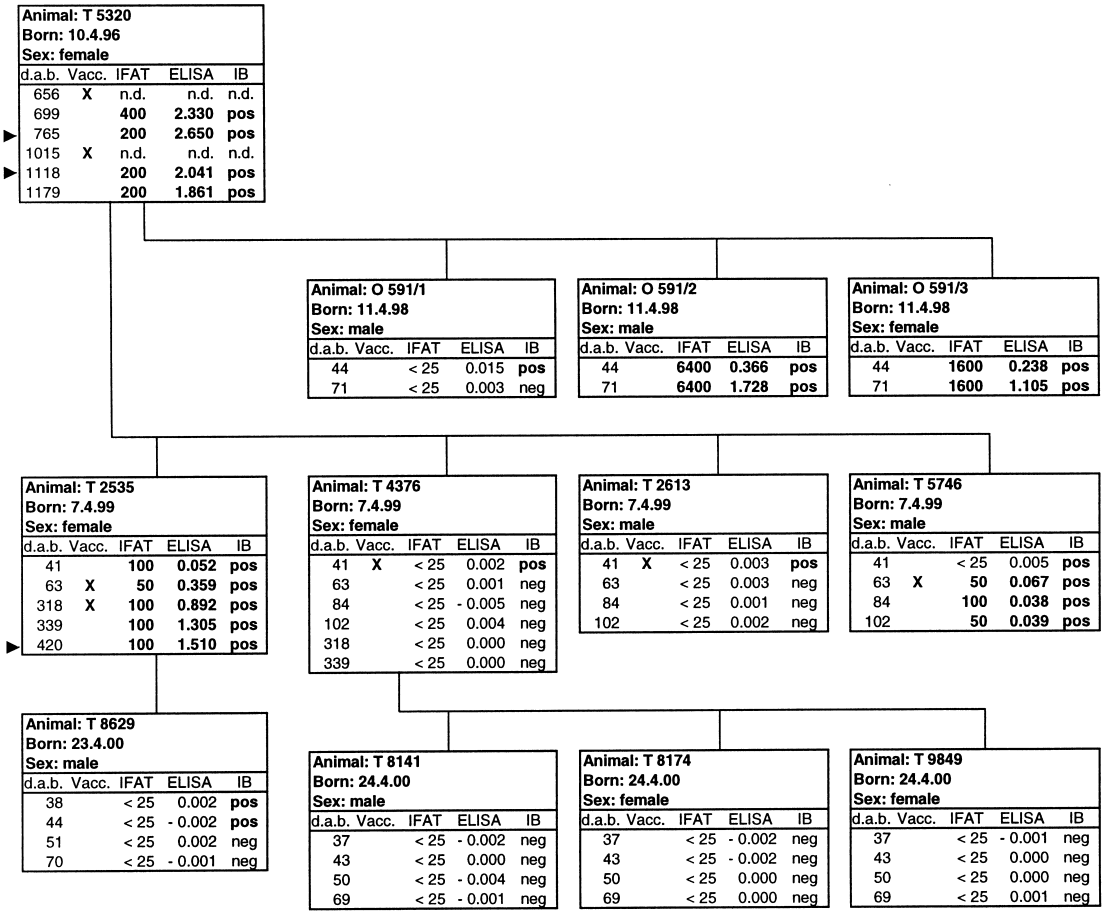

Fig. 3. Genealogical tree of foxes born on a fur farm. The ancestor is fox T5320, a naturally *Neospora caninum*-seropositive animal. The sampling dates are indicated as days after birth (d.a.b.). The serological results regarding *N. caninum*-antibodies as obtained by IFAT, ELISA and immunoblot (IB) are displayed for each sampling date. Positive or inconclusive results are indicated by bold letters. Vaccination with rabies vaccine is indicated by (**X**). The first sampling dates of foxes T5320 and T2535 after parturition are marked (►). N.d., no data; Pos, positive; Neg, negative.

response of their mothers around this time (37–41 days after whelping; Fig. 4).

In the IB, three of four investigated cubs with a persistent antibody response showed stronger reactions than cubs with transient antibody responses on days 38–44 after birth (Fig. 4). While the reactions of cubs with a transient response were mainly directed against the immunodominant antigens of 30, 29, 33 and 37 kDa, further reactions against a variety of antigens were visible in cubs with a persistent response. Cubs O591/2 and O591/3 initially showed strong reactions against antigens of *Mr* 56 and 68 kDa (Fig. 4, lanes c and d). On day 71 after birth, their reactions against the 68 kDa antigen had become faint, and the reactions against the 56 kDa antigen were as strong as before (IB not shown). In the case of T2535, reactions against the 56 and 68 kDa antigens (Fig. 4, lane i) were diminished from day 63 onwards and were no longer visible 318 days after birth (68 kDa; IB not shown) or still visible but very faint on day 420 after birth (58 kDa; Fig. 4, lane j). Cub T5746 initially reacted with the 58 kDa antigen (Fig. 4, lane h), but reactions were no longer visible 84 days after birth (IB not shown). The mother of the persistently positive cubs showed no prominent reactions against the 56 and 68 kDa antigens (Fig. 4; lanes a and e). However, both mothers of positive cubs showed strong responses against an immunodominant antigen of *Mr* 17 kDa, while the cubs showed either a very faint (Fig. 4; lane g, k) or no reaction against this antigen.

4. Discussion

Results from seroprevalence studies suggest that foxes can be natural intermediate hosts of *N. caninum* (Barber et al., 1997; Buxton et al., 1997; Simpson et al., 1997). The purpose of our study was to characterise the antibody response of experimentally infected foxes and to compare these findings with those observed in naturally seropositive (putatively infected) foxes to confirm that foxes can be natural intermediate hosts of *N. caninum*.

After experimental infection with the canine *N. caninum* isolate NC-1, the foxes mounted *N. caninum*-specific antibody levels as determined by ELISA and IFAT similar to those described for cattle (Schares et al., 2000). In the IB, experimentally infected foxes recognised the immunodomi-

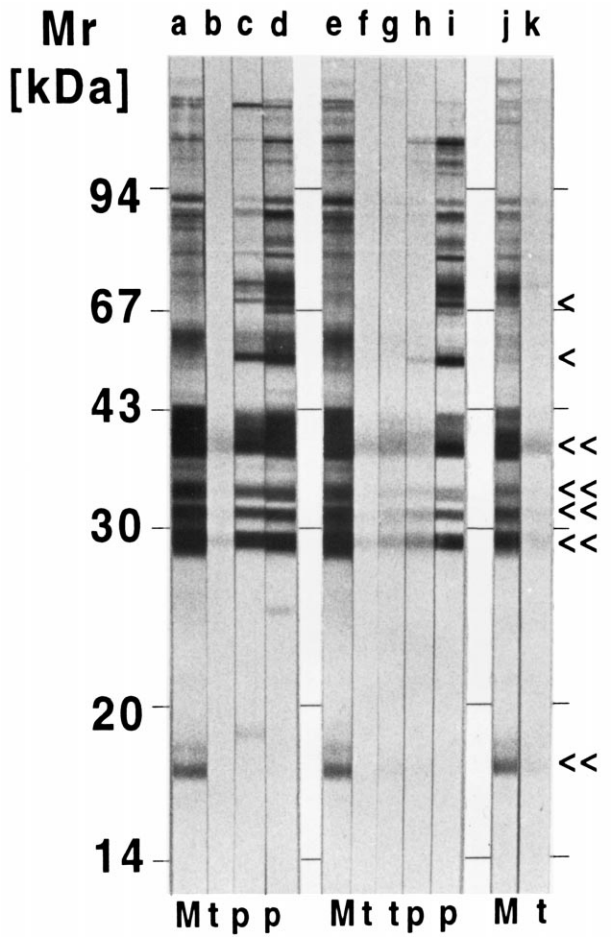

Fig. 4. Comparative immunoblot analysis of sera (sampled 38 and 44 days after birth) from cubs with transient (t) or persistent (p) antibody response against *Neospora caninum*, and sera of their mothers (M) sampled 37–44 days after whelping. Cubs from: (b–d), litter 1 (O591/1, 2, and 3, respectively); and (f–i), litter 2 (T4376, 2613, 5746, and 2535, respectively) were born by vixen T5320 in 1998 (a) and 1999 (e), respectively. Vixen T2535 (j) gave birth to cub T8629 (k) in 2000. The reactions with immunodominant antigens of *Mr* 17, 29, 30, 33 and 37 kDa are indicated (<<). Reactions of sera with antigens of *Mr* 56 and 68 kDa are marked (<).

nant antigens of *Mr* 17, 29, 30, 33 and 37 kDa as has been previously reported for several other species (Barta and Dubey, 1992; Bjerkas et al., 1994; Paré et al., 1995). They also reacted with three antigens of *Mr* 56, 68 and >94 kDa. These reactions seemed to be associated with a period of 14 days–2 months after experimental infection.

We had the opportunity to analyse the time course of the antibody response of naturally *N. caninum*-seropositive foxes on a fur farm. The IB patterns of the naturally seropositive foxes revealed a high degree of similarity to those observed in experimentally infected foxes and resembled those reported for other intermediate host species. We believe that this is sufficient evidence to state that at least some of the members of this fox family had been naturally infected with *N. caninum*.

Neospora caninum infections by the ingestion of tissues of infected intermediate hosts are not expected to occur often on this fur farm since the foxes are fed on sea-fish and poultry meat (chicken, turkey) and have no access to tissues of natural intermediate hosts of *N. caninum*. Infection by oocyst contamination is unlikely since dogs have no access to the farm and bore well water is used for watering. However, occasional postnatal infections by oocyst contamination or by the ingestion of tissues of infected intermediate hosts cannot be excluded. Whether birds are natural intermediate hosts for *N. caninum* is not known, but domestic pigeons have been experimentally infected with *N. caninum* (McGuire et al., 1999). Therefore, poultry meat can not be excluded as a source of infection. Vixen T5320 had been imported from a fox farm in Poland in 1996, where, at that time, the foxes had also been fed on beef. Therefore, it is possible that vixen T5320 had acquired the infection on this farm.

The seropositive vixen and her progeny were among those animals from the fur farm that had been used to analyse the role of maternal antibodies in rabies vaccination. An influence of rabies vaccination on the *N. caninum*-specific antibody response of foxes examined in our study can be excluded since both seropositive and a large number of seronegative foxes had been vaccinated (only partial data shown).

In the retrospective study of the new-born foxes, we found that the *N. caninum*-specific antibody response of cubs born to naturally seropositive vixens displayed different time courses. Some showed positive antibody reactions against *N. caninum* throughout the entire sampling period, indicating that they had been congenitally infected with *N. caninum* or were infected shortly after birth. The transient reactions in other cubs suggest the presence of passively acquired maternal antibodies rather than the presence of an active antibody response against *N. caninum*.

Two findings suggest a recent *N. caninum* infection in the cubs with persistent antibody response: (i), the sera of such cubs showed reactivity with immunodominant antigens which was associated with a recent experimental infection (antigens of *Mr* 56 and 68 kDa), while all cubs with a transient antibody response failed to recognise these antigens; and (ii), on the same sampling date, the mothers showed no or faint reactions with the 56 and 68 kDa antigens. These results clearly indicate that the response is mounted by the cubs themselves.

Since the cubs were first bled between 38 and 44 days after birth, it remains unclear whether cubs with a persistent antibody response were infected congenitally or postnatally. We believe the most likely source of postnatal infection to be the excretion of *N. caninum* stages by their mothers, possibly with the milk. If foxes are definitive hosts of *N. caninum*, oocysts shed by their mothers are other possible sources of infection for the cubs. However, until now, it is not known that foxes can be definitive hosts of *N. caninum*. Other sources of infection which are not directly linked to the mothers appear unlikely, since some of the other foxes should then also have been found to be seropositive.

Symptoms of illness which could be regarded as typical for neosporosis (e.g. neuromuscular disorders) have not been observed on the farm. However, the behaviour of the putatively infected seropositive vixen T2535 who had killed nearly all her cubs needs to be considered. The seropositive vixen T5320 also killed all her cubs in 1997. It is not unusual that vixens kill diseased and abnormal cubs (U. Wenzel, unpublished). Therefore, further studies with more infected vixens are needed to address the question of whether *N. caninum* infection has an influence on cub survival.

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