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ORIGINAL PAPER

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Precolostral serology in calves born from *Neospora*-seropositive mothers

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Abstract The present study was designed to exploratively determine (a) how many healthy calves, born from seropositive mothers, were also precolostrally seropositive; (b) how many precolostrally negative calves became postcolostrally positive; and (c) in these calves, how the IgG1/IgG2 situation developed pre- and postcolostrally. All calves were born from mothers that were determined to be seropositive in a conventional *Neospora caninum*-ELISA and by immunoblotting. When the diagnostic performance of the conventional ELISA was compared with that of immunoblotting and an IgG1/IgG2-ELISA in the calves, the latter two exhibited a higher sensitivity: From a total of 15 precolostral calf sera, 7 were positive in the conventional ELISA (diagnostic sensitivity 47%) compared to 15 that were positive by immunoblotting (diagnostic sensitivity 100%) and 12 that were positive by the IgG1/IgG2-ELISA (diagnostic sensitivity 80%). With regard to IgG1/IgG2 findings in the dams, IgG2 appeared as the dominant subclass in the humoral immune response of adult cattle, while in calves, IgG1 appeared as the main prenatally/precolostrally reactive antibody isotype. Provided that precolostral seropositivity reflects postnatal persistent infection with *N. caninum*, we then postulate that, basically, all of our study calves born form *N. caninum*-seropositive mothers were prenatally infected with the parasite, and may, thus, all become members of the next transmitting generation.

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Introduction

The apicomplexan protozoa *Neospora caninum* is one of the most frequently diagnosed causes of infectious abortions in cattle worldwide. Neosporosis results in neuromuscular disorders, paralysis, and death in dogs, whereas abortion and neonatal morbidity is the main characteristic in cattle. *Neospora caninum* in cattle represents an economically important health problem with considerable impact on the livestock industry (Hietala and Thurmond 1999; Hernandez et al. 2002). In Switzerland, between 25 (Sager et al. 2001) and 29% (Gottstein et al. 1998) of abortions have been associated to *N. caninum* infections based on molecular (PCR) and pathohistological assessment of the abortion cause. Currently, there is no effective method available that allows the effective and efficient control of the problem. Vaccination has been suggested to prevent neosporosis in cattle; thus, different approaches to develop an effective vaccine have been tackled (Innes et al. 2002). However, Andrianarivo et al. (2000) reported on the failure of the vaccine to protect herds from abortion. Little information is available on the metaphylactic use of drugs to address the problem. An experimental chemotherapeutical treatment study found that toltrazuril medication in female C57/BL6 mice could considerably reduce the diaplacental passage of the parasite to the fetal brain (Gottstein et al. 2005). An explorative study to assess the efficacy of toltrazuril-sulfone (ponazuril) has been carried out in calves experimentally infected with *N. caninum* (Kritzner et al. 2002).

Epidemiologically, the endogenous (vertical) transmission was shown to occur in approximately 81–90% of cases (Wouda 2000). This most important route of transmission is mainly responsible for the maintenance or spread of an infection in a herd by means of successive passage from generation to generation (Dubey 1999). Most congenitally acquired infections also result in the birth of apparently healthy calves that become the new carriers. The incidence of abortion is basically a rare event and has been estimated to range between 2 and 5% (Dubey 1999; Hemphill and Gottstein 2000; Wouda 2000).

Seroepidemiological analysis in affected herds demonstrated a statistical association between seropositivity and abortion (Thurmond and Hietala 1997; Otranto et al. 2003; Piergili Fioretti et al. 2003), in that the incidence of abortion in seropositive cows appeared approximately four times higher than that in seronegative cows (Davison et al. 1999; Sager et al. 2001; Haessig and Gottstein 2002). Other groups claimed a protection by high antibody concentrations against abortion due to *N. caninum* (Innes et al. 2001; Williams et al. 2003; Guy et al. 2001). Experimental infection of pregnant cows at various time points during gestation showed that parasitemia during the first 10 weeks resulted in embryopathy and resorption of the embryonic tissue 3 weeks after infection. Infection at 30 weeks of gestation resulted in the birth of asymptotic, congenitally infected calves (Williams et al. 2000). The period in-between 10 and 30 weeks gestation appears to be that most likely for *N. caninum*-induced abortion. Infection with *N. caninum* gives rise to an antibody response, which can be demonstrated by different tests. The presence of antibodies in an animal principally indicates that it is, or has recently been, infected with the parasite, but is not proof to directly associate infection with the abortion event. In cattle, *N. caninum* antibodies fluctuate during pregnancy, and also in-between pregnancies, and they may, at least with some assays, even drop below the detection limits (Kyaw et al. 2005; Staubli et al. submitted for publication).

In a perinatal situation, serology in the newborn calf and its mother requires a special consideration in that a selective transfer of IgG1 from serum to colostrum occurs in the mother just before parturition. Thus, the precolostral serum in a calf may provide a good indication of whether the animal has been exposed to *N. caninum* in utero (Bartley et al. 2004), whereas postcolostral serology will be influenced by the maternal antibody transfer via milk (Erhard et al. 2001).

The questions addressed in the present study were: (a) how many healthy calves born from seropositive mothers were precolostrally positive (thus indicating prenatal infection); (b) how many precolostrally negative calves became postcolostrally positive; and (c) were there pre- and postcolostral peculiarities with regard to IgG1/IgG2 dichotomy, anticipating that the colostral input focuses on the IgG1 isotype.

Materials and methods

Neospora caninum parasite

The *N. caninum* strain NC-LIV (GB) was used to generate tachyzoites in vitro that were harvested by trypsinization of the Vero cell monolayers. Suspensions of tachyzoites with cellular debris were sedimented at 1,000×*g* for 10 min. The pellet was resuspended in phosphate-buffered saline (PBS, pH 7.2), passed through a 25-gauge needle at 4°C, and subsequently separated on a DP10 column filled with Sephadex G-25 (Pharmacia), as described by Hemphill et al. (1996). The purified tachyzoites were stored as a semidry pellet at −80°C until use. Frozen tachyzoites were used to prepare the immunoblot-antigen exactly as described by Schares et al. (1998, 1999).

Neospora caninum somatic antigen ELISA

The conventional IgG-detecting ELISA system was employed as previously described by Gottstein et al. (1998). The negative/positive threshold value was defined by the mean plus three standard errors of 30 sera from cows repeatedly negative in the *Neospora*-indirect fluorescent-antibody test and -ELISA and that were derived from farms exhibiting no *Neospora* problems and very low or negative seroprevalence for *N. caninum*. The positive control serum was from an experimentally infected calf (Kritzner et al. 2002). Positive serological ELISA results were expressed in percentual antibody reactivity (%AR) obtained by linear regression between the cut-off value and that of a highly positive control serum (Gottstein et al. 1998).

For the test performance, frozen antigen plates obtained from Bommeli AG (Bern) were thawed at room temperature. Test and control sera were used at a dilution of 1:100. The second antibody (CHEKIT-Neospora-Anti-Ruminant-IgG-PO-Konjugate, monoclonal, marked with horseradish-peroxidase, Bommeli AG Bern) was added. Antibody reactivity was detected by adding the CHEKIT-TMB substrate (Bommeli AG Bern). Absorbance values were read at $A_{450\ nm}$ using a Dynex MRX II photometer.

Additionally, an isotype-specific *N. caninum* ELISA was carried out, including the differentiation between antigen-reactive IgG_1 and IgG_2 subclasses. For this ELISA, the basic parameters were as described above, with the following modifications: The second anti-IgG1 antibody (alkaline phosphatase-conjugated sheep anti-bovine IgG1;1 mg/ml, Betyhl) was used at a dilution of 1:50, and the anti-IgG2 antibody (alkaline phosphatase-conjugated sheep anti-IgG2, 1 mg/ml, Bethyl) was diluted 1:100. The antibody reactivity was detected by adding 4-nitrophenylphosphate at a concentration of 1 mg ml^{-1} in 0.1 M diethanolamine, pH 9.8. Absorbance values were read at $A_{405\ nm}$ (reference $A_{630\ nm}$) using a Dynex MRX II photometer. The determination of the negative/positive threshold in the IgG1 and the IgG2 ELISAs, respectively, was individually carried out for dam and calf sera by calculating the mean ELISA value plus 4 SD of 30 negative dam or calf sera, respectively. Any values higher than these cut-offs were considered to be "positive" and lower values were considered as "negative." Semiquantifications of ELISA reactions were done as described for the conventional ELISA above, data were expressed in percentual relative antibody reactivity (%AR) obtained by linear regression between the cut-off value and that of a highly positive control serum (Gottstein et al. 1998).

Neospora caninum immunoblot

The immunoblot procedure was basically carried out as described by Schares et al. (1998, 1999) and Staubli et al. (submitted for publication). All sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) chemicals were obtained from Fluka (Fluka Chemie, Buchs, Switzerland). The frozen parasite pellets, each containing 0.66×10^7 *N. caninum* tachyzoites, were resuspended in 80 µl of sample buffer (Schares et al. 1998) and immediately boiled for 5 min at 95°C. The samples (10 µg protein per centimeter slot) were electrophoretically separated in a 12.5% gel with a 3% stacking gel. The SDS-PAGE-resolved components were electrophoretically blotted onto a sheet of polyvinylidene difluoride (PVDF) (Immobilon-P, pore size 45 µm, lot no. K4HN4464H). Cut PVDF strips were exposed to sera diluted 1:100 with PBS containing 0.05% Tween20 and 2% fish gelatine liquid (PBS-TG) (Serva No 22156). The strips were incubated for 1 h with gentle rocking on a prewarmed thermoelement (37°C). They were subsequently washed six times with PBS-TG prior to incubation with anti-bovine IgG (H+L) peroxidase conjugate (Dianova, catalog number 101-035-003) used at a dilution of 1:300 in PBS-TG for 1 h by gentle rocking on a prewarmed thermoelement. The strips were washed four times with PBS-TG and two times with PBS only. Antigen–antibody-reactive bands were visualized using 4-chloro-1-naphthol (Sigma, C-6788) as a precipitating chromogenic substrate. Prestained standard protein ladder markers were from BioRad (catalog number 161-0374Rad). The marker section and the first 5 mm of the *N. caninum*-antigen part of the PVDF membrane were stained with found india ink (Pelikan) solution as described by Hancock and Tsang (1983). To identify antibody reactions against the relevant immunodominant antigens, the protocols described by Schares et al. (1998; 1999) and (Söndgen et al. 2001) were exactly followed, which were principally based upon the detection of at least two specific bands per strip.

Mother and pre- and postcolostral calf sera

Precolostral calf sera (born from seropositive mother cows) were obtained by umbilicordal punction. The postcolostral sera were obtained by venipuncture 72 h after feeding the calves with colostrum.

The animals investigated were grouped as follows:

Group 1 Sera from ten (*N. caninum*-ELISA and *N. caninum* immunoblot) serologically negative control dams and corresponding calves were derived from the paired serum library as described in group 2 (see below). These control sera were used to determine the negative/positive threshold in the IgG1 and the IgG2 ELISAs, respectively.

Group 2 Sera from 36 dam–calf pairs were obtained from the Department of Farm Animals, University of Zürich. These samples were collected directly at delivery between 2001 and 2003 in farms located in the vicinity of the Veterinary Faculty of Zürich. These farms had participated in a noninfectiological study on fertility performances, and the respective samples had been stored frozen at −30°C. Preselection criteria for the present study were a healthy newborn calf and an appropriately reliable precolostral serum sampling carried out by the investigator (M. Haessig).

Group 3 Sera from eight dam–calf pairs were selected from farms located in the eastern part of Switzerland. Preselection criteria for the present study were a persistently seropositive mother animal (*N. caninum*-

Table 1 Individual serological testing of dam sera (numbers 297, 517, 519, 520, 1342, 1345, 1916) and corresponding precolostral calf sera (numbers 298, 516, 521, 522, 1343, 1346, 1931) by conventional ELISA, immunoblotting, and IgG1/IgG2-isotype ELISA

Number	ELISA[a] (D)	ELISA[a] (precC)	Immunoblot	IgG1[a] (D)	IgG2[a] (D)	IgG1[a] (precC)	IgG2[a] (precC)
297	11		pos	1	21		
298		0	pos			0	0
517	92		pos	18	25		
516		0	pos			0	3
519	17		pos	0	0		
521		0	pos			0	1
520	26		pos	0	0		
522		0	pos			0	0
1342	36		pos	3	43		
1343		20	pos			16	5
1345	56		pos	13	77		
1346		39	pos			15	4
1916	53		pos	8	100		
1931		0	pos			0	0

D dam sera, *precC* precolostral calf sera, *pos* positive

[a]ELISA values are expressed as percentual relative antibody reactivity (%AR) obtained by linear regression between the cut-off value and that of a highly positive control serum (Gottstein et al. 1998); a 0 value refers to a seronegative serum

Table 2 Individual serological testing of dam sera and corresponding calf sera, sampled precolostrally or postcolostrally

Number	ELISA[a] (D)	ELISA[a] (precC)	ELISA[a] (postcC)	Immunoblot (D)	Immunoblot (precC)	IgG1 (D)	IgG2 (D)	IgG1 (precC)	IgG2 (precC)	IgG1 (postcC)	IgG2 (postcC)
18369 (D)	63			pos		19	35				
18371 (C)		45			pos			32	26		
18370 (C)			104							68	30
18599 (D)	1			pos		2	21				
18601 (C)		0			pos			2	8		
18600 (C)			82							8	18
18984 (D)	60			pos		27	17				
18985 (C)		113			pos			77	9		
18986 (C)			149							73	14
19213 (D)	22			pos		17	34				
19215 (C)		0			pos			24	6		
19214 (C)			96							13	38
19249 (D)	20			pos		18	21				
19251 (C)		0			pos			3	2		
19250 (C)			74							28	9
19260 (D)	67			pos		23	19				
19262 (C)		47			pos			41	19		
19261 (C)			147							47	18
19312 (D)	59			pos		13	21				
19314 (C)		10			pos			33	43		
19313 (C)			130							51	59
19381 (D)	3			pos		0	19				
19383 (C)		20			pos			19	15		
19382 (C)			61							25	34

Testing was carried out by conventional ELISA, immunoblotting, and IgG1/IgG2-isotype-ELISA
D dam sera, *C* calf sera, *precC* precolostrally sampled, *postcC* postcolostrally sampled, *pos* positive
[a]ELISA values are expressed as percentual relative antibody reactivity (%AR) obtained by linear regression between the cut-off value and that of a highly positive control serum (Gottstein et al. 1998); a 0 value refers to a seronegative serum

ELISA and *N. caninum* immunoblot) till delivery and a healthy newborn calf with an appropriately reliable precolostral serum sampling carried out by the investigator (C. Haerdi). All calves were also postcolostrally sampled at different postparturient time points.

Results

Group 1 Paired serum samples from group 2 (see below) that were negative in the conventional *N. caninum*-ELISA and also negative in immunoblotting were used to calibrate the negative–positive thresholds for the IgG1 and IgG2 ELISAs. In terms of absolute absorbance values, pretesting of sera had indicated that pre- and postcolostral calf sera expressed lower background reactivities than the corresponding negative dam sera. This required the calculation of individual negative–positive cut-off values for the two individual serum groups (data not shown).

Group 2 In the first step, all 36 dam sera of the dam–calf pairs were tested with the conventional *N. caninum*-ELISA detecting bovine IgG and immunoblotting. With regard to these dam sera, ELISA-positivity matched immunoblot-positivity in seven cases, the remaining 29 dams were negative in both assays. When subsequently addressing the paired samples, two of them reacted positive in the ELISA; in five cases only the dam was ELISA-positive, while the precolostral calf serum was seronegative (Table 1). The immunoblotting assay showed that all of the seven pairs yielded positive findings, also including the five precolostral calf sera that had been negative by the conventional ELISA. The same seven serum pairs were additionally tested in IgG1 and IgG2 ELISAs (Table 1). Five out of the seven dams showed an IgG2 antibody reactivity higher than that of IgG1, while in two dams, both IgG1 and IgG2 detection remained negative. For the precolostral calf sera, three groups of reactivity were found. One included three calves that were completely negative for both antibody subclasses; the same sera had been negative in the conventional ELISA. Two calves were weakly positive for IgG2 and negative for IgG1; the respective conventional ELISAs were negative. Two other calves, initially positive in the conventional ELISA, exhibited a higher antibody reactivity with regard to the IgG1 isotype when compared to IgG2.

Group 3 The eight double-paired sera from dams and calves (pre- and postcolostral samples) were first tested by

conventional *N. caninum*-ELISA and immunoblotting (Table 2). The ELISA antibody reactivity ranged from very low (1 %AR) to high (67 %AR). All dams were also positive by immunoblotting. Five dams exhibited higher IgG2 than IgG1 reactivity in ELISA, one dam was negative for IgG1 and positive for IgG2, while two dams were slightly higher in IgG1 than in IgG2 reactivity. All postcolostral calf sera scored positive in the conventional ELISA, whereas three out of eight precolostral sera were negative. In all cases, the postcolostral sera exhibited a markedly higher antibody reactivity than the precolostral sera, thus indicating a successful colostrum uptake. IgG1 and IgG2 ELISAs were positive for all pre- and postcolostral calf samples. In six out of eight precolostral samples, IgG1 reactivity was higher than that of IgG2. Postcolostrally, a rise in antibody reactivity was observed in six out of eight calves for IgG1 and in seven out of eight cases for IgG2.

Discussion

Vertical transmission is a very important aspect of the asexual life cycle of *N. caninum* because it may keep prevalences nearly constant within a cattle population without a need for dispersion by the definitive host (Schares et al. 1998). Therefore, the question addressed by this study was to determine how many healthy calves born from *N. caninum*-seropositive dams were precolostrally positive, as determined by different test systems, and how many became postcolostrally positive. In the same frame of this study, we also tackled the IgG1/IgG2 dichotomy of parasite-specific antibodies, anticipating that IgG1 would by the main isotype to be colostrally transferred to the offspring. In fact, the ruminant placenta does not permit the transfer of maternal immunglobulins to the fetal circulation (Quigley and Drewry 1998). Consequently, parasite-specific antibodies detectable in precolostral sera have most likely been prenatally synthesized by the fetus, indicating an active immune response against the invading parasite. This is usually feasible during the last 3 months of gestation. After birth, during the first 24–36 h of life, the calf is able to absorb immunglobulins from the mother's milk through the gut, and, consequently, antibodies with specificities identical to those found in the mother appear within the serum of the calf (Stott et al. 1979). The major antibody subclass found in colostral milk is IgG1, whereas in serum, both IgG1 and IgG2 are present; a selective transfer of IgG1 from serum to colostrum occurs in the mother just before parturition. Thus, the precolostral serum sample may provide a good indication of whether the animal has been exposed to *N. caninum* in utero. In the case of a healthy born calf, this may strongly indicate that the calf will become a persistently infected new animal. In case of seronegativity, one may postulate that the calf could have been born as a parasite-free offspring. Although one may claim that infection at a very early stage of gestation may fall within a phase of immunotolerance, and thus, incompetence to react immunologically; infection at that stage is at high risk for resulting in abortion, at least as experienced in ovine and human toxoplasmosis. Nevertheless, we still cannot exclude that persistently infected seronegative animals are at risk, as found, e.g., in bovine virus diarrhea viral infections.

The methodical reliability of the precolostral serology is therefore a crucial component in addressing our question. Based upon previous experiences (Staubli et al. submitted for publication), we realized that immunoblotting may present a better diagnostic sensitivity than conventional ELISAs, such as described by von Blumroder et al. (2004). Therefore, we tested all of our samples in parallel by ELISA and immunoblotting. By conventional ELISA, approximately 47% (seven out of 15 calves in all) only of newborn animals were serologically positive by conventional ELISA. This may lead to the interpretation that the congenital transmission rate is 40%. Now, conversely, all calves were precolostrally seropositive by immunoblotting. This now indicates that 100% of these animals must have had intrauterine contact with the parasite, including a respective seroconversion, which indicates that, in all of these cases, a congenital transmission must have occurred. Consequently, immunoblot analysis of precolostral sera appears mandatory to carry out appropriate studies. When addressing the isotype-specific ELISA serology, we realized that 12 out of 15 calves (80%) were seropositive at least in one of the assays. This could be explained by the fact that, for these two tests, we had individually determined respective threshold values for calves and dams. This optimization allowed us to increase the diagnostic sensitivity for the calf groups. Consequently, in future studies we will also adapt the cut-off calculations of the conventional ELISAs for each age group, as already suggested by others (von Blumroder et al. 2004; Schares et al. 2005).

With regard to IgG1/IgG2 findings in the dams, IgG2 appears as the dominant subclass in the humoral immune response of adult cattle. In calves, however, although there were only 10 out of 15 animals positive for IgG1—comparative to 12 out of 15 IgG2-positives—the relative antibody concentration itself appeared higher for IgG1 (mean %rAU=17.5) than for IgG2 (mean%rAU=9.4). Little is known about the specific differential role of IgG1 vs IgG2 in bovines. We may anticipate that both isotypes are involved in controlling the course of infection. At least prenatally synthesized antibodies (detected in the precolostral serum) reflect the intrauterine contact with *N. caninum* on one hand, but, obviously, also a successful host–parasite interaction, in so far as the calf was subsequently born healthy. We do not know if in all cases the parasite survives postnatally, and also we do not know if the parasites are still all tachyzoites at the time point of delivery, or if some have already differentiated into bradyzoites. In a healthy born calf, it is practically impossible to demonstrate the presence of the parasite by histology or PCR, especially due to the low infection intensity encountered in this situation. Epidemiological data, however, clearly show that most of the calves remain persistently infected and, if used for breeding purposes,

will become the next generation of *N. caninum* endogeneously transmitting individuals.

Overall, our results suggest that, beside immunoblotting, IgG1/IgG2-ELISAs also exhibit higher sensitivity than conventional IgG-ELISA when investigating precolostral samples, most likely due to the individual adaptation of the cut-off values to individual animal age groups. Thus, both complementary tests appear suitable in detecting *N. caninum* infections in cattle, and their use may especially be indicated in critical cases such as precolostral investigations where highly sensitive tools are required. In terms of our findings, we postulate that, in our study, all of the calves born form *N. caninum*-seropositive mothers were already prenatally infected with the parasite, and thus, may all become members of the next transmitting generation.

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