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ORIGINAL PAPER

Neospora caninum NC-6 Argentina induces fetopathy in both serologically positive and negative experimentally inoculated pregnant dams

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Abstract *Neospora caninum* infection is a major cause of abortion in cattle. The objectives of this study were to genetically characterize the *N. caninum* NC-6 Argentina isolate using a multilocus microsatellite analysis approach and to study its biological behavior by experimental inoculations into seronegative and seropositive pregnant cattle, evaluating the humoral and cellular immune response elicited and the occurrence of transplacental transmission and fetopathy. Pregnant cows (65 days of gestation) seropositive and seronegative to *N. caninum* were intravenously inoculated with tachyzoites of the NC-6 Argentina *N. caninum* strain and slaughtered at 108±2 days of gestation. Serum samples were analyzed for *N. caninum* antibodies by indirect fluorescent antibody test. The cellular immune response was analyzed by detection of gamma interferon (γIFN) production in blood cells. Tissue samples from dams, fetuses, and placental cotyledons were processed by histopathological and immunohistochemical techniques and examined for *N. caninum* DNA by PCR. Positive DNA samples were further analyzed by multilocus microsatellite typing for *N. caninum*. Inoculated animals had significantly higher *N. caninum* antibody titers and γIFN production than control animals. One seropositive inoculated cow aborted, one seronegative cow had a non-viable fetus, and the remaining fetuses from the experimentally inoculated dams had histopathologic lesions. The PCR was positive in 3/4 fetuses from seronegative inoculated cows and in 2/3 fetuses from seropositive inoculated cows. Multilocus microsatellite analysis revealed that the *N. caninum* DNA present in fetuses and placentas had an identical pattern to NC-6 Argentina strain. The NC-6 Argentina strain proved to be able to cross the placenta and to induce fetopathy in both the seropositive and seronegative dams.

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Introduction

Neospora caninum is an apicomplexan protozoan parasite which represents a major cause of abortions in cattle worldwide and of neuromuscular disease and death in dogs. Numerous studies confirmed the association between *N. caninum* infection and abortions, particularly in dairy cattle (Thilsted and Dubey 1989; Anderson et al. 1991; Venturini et al. 1999; Dubey 2003a; Barr et al. 1994; Gottstein et al. 1998).

Bovines can acquire the infection horizontally, through ingestion of oocysts shed with the feces of canids, the definitive hosts (Mc Allister et al. 1998; Basso et al. 2001; Gondim et al. 2004) or, vertically, by transplacental transmission from

the dam to the fetus (Dubey 2003a, b; Paré et al. 1996; Hall et al. 2005; Davison et al. 1999; Moré et al. 2009). Vertical transmission is highly efficient (Paré et al. 1996; Davison et al. 1999), and the consequences include abortion, birth of weak calves that die shortly after birth, or birth of clinically healthy but infected calves that may transmit the infection to their offspring in consecutive pregnancies (Innes et al. 2002). The economic losses caused by bovine neosporosis include costs of reproductive failure, professional care and diagnosis, premature culling of seropositive animals, and decreased milk production (Hall et al. 2005; Dubey 1999; Anderson et al. 2000; Thurmond and Hietala 1996, 1997).

Detection of antibodies to *N. caninum* indicates exposure to the parasite, or presence of maternal antibodies in young calves that ingested colostrum. The indirect immunofluorescence test (IFAT) was the first test employed to detect *N. caninum* antibodies and has been used as the reference test for development of other serological tests (Björkman and Uggla 1999; Jenkins et al. 2002; Dubey and Schares 2006). However, the effectiveness of the antibody response against *N. caninum* is limited to the form of extracellular tachyzoites, while the cellular immune response plays a major role in the protection against this intracellular parasite (Innes et al. 2002, 2005; Williams et al. 2007). Detection of gamma interferon (γIFN) is a good indicator of cellular immunity. In aborted fetuses, histopathological and immunohistochemical (IHC) examination of the organs is needed to establish neosporosis as the cause of abortion (Dubey 2003b; Campero et al. 1998).

N. caninum isolation is difficult because samples from animal tissues are often autolyzed or contaminated. In Argentina, Basso et al. (2001) first isolated *N. caninum* from oocysts found in feces of a naturally infected dog. This strain was called NC-6 Argentina, but so far, little is known about the genetic and biological characteristics of this isolate. Knowledge concerning biological characteristics of different isolates such as virulence, ability for transplacental transmission, and effect on the host immune response is of primary importance for the design of vaccines. Recent studies showed that *N. caninum* has a wide genetic diversity, so differences in biological behavior could be also expected. Microsatellite–DNA sequence analysis proved to be a valuable tool to study this genetic diversity (Al-Qassab et al. 2009, 2010; Basso et al. 2009a, b, 2010; Beck et al. 2009; Pedraza-Diaz et al. 2009; Regidor-Cerrillo et al. 2006, 2008; Rojo-Montejo et al. 2009). Microsatellites or simple sequence repeats are highly variable loci which consist of tandemly repeated units of 1–6 bp length, present in the genome of eukaryotic and prokaryotic organisms. The polymorphisms in these sequences result from the gain and loss of single repeat units. Multilocus microsatellite typing can be achieved either by sequencing or by assessing the length of the amplified microsatellite containing fragments by capillary electrophoresis using fluorescent-labeled primers. *N. caninum* microsatellite amplification by nested PCR showed a higher sensitivity than by conventional PCR (Basso et al. 2009b; Pedraza-Diaz et al. 2009).

The aims of this study were to genetically characterize the *N. caninum* NC-6 Argentina isolate using a multilocus microsatellite analysis approach and to study its biological behavior in experimentally infected pregnant cattle, evaluating the humoral and cellular immune response elicited and the occurrence of transplacental transmission and fetopathy both in seronegative and seropositive animals. Furthermore, we wanted to evaluate the practical use of multilocus microsatellite analysis in experimental infections in cattle, tracing *N. caninum* isolates with a known genetic background.

Materials and methods

Animals and experimental design

Eighteen pregnant Aberdeen Angus cows, free of toxoplasmosis, brucellosis, tuberculosis, campylobacteriosis, and trichomonosis, were utilized for this study. Free status for tuberculosis and bovine brucellosis was tested by intradermal purified protein derivative test and buffered plate antigen test, respectively, according to Argentinean sanitary resolutions (SENASA Res. 115/1999). Diagnosis for *Campylobacter fetus* and *Tritrichomonas* fetus from cervicovaginal samples were performed by culture and immunofluorescence assay as described (Campero et al. 2003). Serum samples from cows were examined by IFAT for specific antibodies against *Toxoplasma gondii* as described (Moré et al. 2008). Vaccination with inactivated vaccines against bovine viral diarrhea virus and bovine herpesvirus-1 (Biogenesis Lab., Argentina) was performed twice 2 months before breeding. At the beginning of the study, the cows were tested for specific antibodies to *N. caninum* by the IFAT from 1:25 dilution (Moré et al. 2008, 2009) and divided in four groups:

Group 1 Seronegative (IFAT titer <1:25), non-inoculated control animals (SNC): cows nos. 608 and 2071

Group 2 Seronegative (IFAT titer <1:25) animals inoculated with NC-6 Argentina strain (SN-NC6): cows nos. 257, 603, 989, and 2005

Group 3 Seropositive (IFAT titer ≥1:100), non-inoculated control animals, naturally infected with *N. caninum* (SPC): cows nos. 220 (IFAT titer 1:3,200), 229 (IFAT titer 1:800), 241 (IFAT titer 1:800), and 299 (IFAT titer 1:1,600)

Group 4 Seropositive (IFAT titer ≥1:100) animals, naturally infected with *N. caninum*, inoculated with NC-6 Argentina strain (SP-NC6): cows nos. 265 (IFAT titer 1:1,600), 269 (IFAT titer 1:400), 242 (IFAT titer 1:3,200), and 298 (IFAT titer 1:1,600)

Cows from groups SN-NC6 and SP-NC6 were inoculated intravenously (iv) with 5×10^7 tachyzoites of NC-6 Argentina isolate at 65 days of gestation. Cows from control groups were iv inoculated with phosphate-buffered saline solution (PBS).

To determine antibody levels, serum samples were collected on days 0 (preinoculation), 5, 11, 18, 24, 32, 39, and 42 post-inoculation (dpi). The cellular immune response was analyzed by detection of γIFN production in blood cells obtained from blood samples collected at 30 and 37 dpi using a commercial capture ELISA. At 108±2 days of gestation, cows were slaughtered and tissue samples from dams, fetuses, and placentas were taken for histopathological, IHC, and molecular analysis.

N. caninum parasites

N. caninum tachyzoites of the NC-1 and NC-6 Argentina strains were in vitro cultured in Vero cells with RPMI medium supplemented with 10 % fetal calf serum, 1 % penicillin, and streptomycin, at 37 °C and 5 % CO_2 atmosphere. When approximately 50 % of the monolayer was disrupted, cells were scraped and the suspension was passed through needles of 20, 22, and 25G and washed twice in PBS by centrifugation at 800×*g*. NC-6 Argentina and NC-1 tachyzoites were used for antigen preparation for IFAT, and only NC-6 Argentina parasites were used to perform the animal inoculations after confirming viability by Trypan blue staining and counting in a Neubauer chamber.

Serologic analysis by IFAT for *N. caninum*

IFAT was performed using either NC-1 or NC-6 Argentina tachyzoites as antigen, as described by Dubey et al. (1988). Bovine sera were tested until final titer beginning with a 1:25 dilution and fluorescein isothiocyanate-conjugated anti-bovine IgG whole molecule (SIGMA, Saint Louis, MO, USA) was used as secondary antibody. Sera with titer ≥1:25 were considered positive. Geometric mean antibody titers (GMAT) were obtained and analyzed by ANOVA.

Detection of γIFN production

Blood samples were collected with EDTA on days 30 and 37 post-inoculation from all cows. One milliliter of each blood sample was cultured in duplicate wells in a 24-well culture dish at 37 °C in a 5 % CO_2 atmosphere and stimulated with NC-1 tachyzoite lysate at a final concentration of 50 μg protein/ml or PBS as negative control. Culture supernatants were collected and analyzed for detection of γIFN production by a capture ELISA test (BOVIGAM, CSL, Parkville, VIC, Australia) following the manufacturer's instructions. Optical densities (OD) were read in a spectrophotometer (Labsystems Multiskan MS, Finland), and the final OD for each sample was obtained by subtracting the OD mean of the negative control minus the OD average of each stimulated sample. A sample was considered positive when the final OD was equal or higher than 0.1.

Histopathological and immunohistochemical analysis

At necropsy, central nervous system (CNS) samples from dams, placental cotyledons, and CNS, heart, tongue, striated muscle, liver, kidney, umbilical cord, and lung samples from fetuses were collected and fixed in 10 % formalin, embedded in paraffin, and routinely processed for histological examination. Sections were cut to 5 μm thickness and stained with hematoxylin and eosin (H&E). The observed lesions were classified according to an arbitrary score, as follows: absent (−), light (+), moderate (++), and severe (+++), based on the severity and number of inflammatory lesions. IHC for *N. caninum* was performed on fetal and placental tissues with the avidin–biotin technique using a commercial kit (Vectastain®, Peroxidase Elite ABC PK-601, Vector Laboratories, Burlingame, CA, USA) according to a previously described protocol (Campero et al. 1998). A rabbit anti-*N. caninum* hyperimmune serum (kindly provided by Dr. M. Anderson, UC Davis, Davis, CA, USA) was used at a 1:300 dilution as the primary antibody, and a goat anti-rabbit IgG was used as a secondary antibody.

PCR for *N. caninum* DNA and multilocus microsatellite analysis

DNA from in vitro-cultured *N. caninum* NC6 Argentina tachyzoites and from tissue samples of fetal brain and heart, placenta, and cow brain were extracted with a commercial kit (QIAGEN Hilden, Germany; DNeasy tissue and blood kit) according to the manufacturer's instructions. All DNA samples were initially subjected to a *N. caninum*-specific PCR with the primer pair Np6/Np21 (Yamage et al. 1996), using DNA from NC-1 strain as positive control and the master mix without DNA sample but water, as negative control. Samples that yielded positive results were further analyzed by multilocus microsatellite typing for the microsatellites MS1B, MS2, MS3, MS4, MS5, MS6A, MS6B, MS7, MS10, MS12, and MS21 of *N. caninum* (Regidor-Cerrillo et al. 2006). Microsatellites MS1B, MS2-4, and MS10 from NC6 Argentina strain were amplified by nested PCR and sequenced using primers and protocols previously described (Basso et al. 2009b). The microsatellites MS5, MS6A, MS6B, MS12, and MS21 were amplified by nested PCR, and the fragment length analysis was performed by capillary electrophoresis (Basso et al. 2010). The microsatellite analysis of *N. caninum* DNA from bovine tissues was performed by nested PCR amplification and sequencing (MS2 and MS10) or fragment length determination by capillary

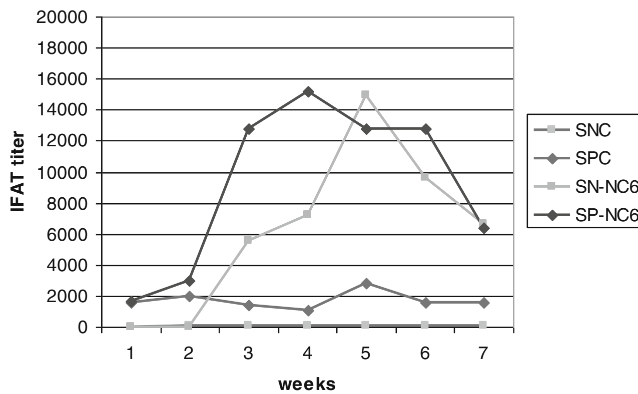

Fig. 1 Weekly evolution of IFAT titers (means) for each group of bovines experimentally inoculated with NC-6 Argentina strain of *N. caninum* and control animals. *SNC* uninfected negative control group, *SPC* uninfected positive control group, *SN-NC6* infected seronegative group, *SN-NC6* infected seropositive group

electrophoresis (MS1B, MS3, MS5, MS6A, MS6B, MS12, and MS21) (Basso et al. 2009b, 2010). DNA from *in vitro*-cultured *N. caninum* tachyzoites of NC-1 and NC-GER6 strains, with known microsatellite sequences (Basso et al. 2009b, 2010), were included in all microsatellite analysis as reference controls.

Results

Humoral immune response

Both seropositive and seronegative cows inoculated with NC-6 strain significantly increased *N. caninum* IgG antibody titers (ANOVA, $p<0.05$; Fig. 1). Seronegative cows (SN-NC6 group) had titers between 1:1,600 and 1:12,800 2 weeks post-inoculation (pi) and reached the maximum titer 4 weeks pi. SP-NC-6 group showed the highest mean antibody titer and an earlier antibody peak compared to SN-NC6 group. One SP-NC6 cow reached a titer of 1:102,400, but this sample was considered an outlier and therefore not taken into account for statistical analysis. Non-inoculated seronegative and seropositive control groups showed no significant variation in antibody titers during the study.

The GMAT for the seronegative control group was significantly different from the others (ANOVA, $p<0.01$), and the same occurred with the seropositive infected group. The GMAT in the SPC group and the SN-NC6 group were similar (Fig. 2). IFAT antibody titers were similar whether *N. caninum* NC-1 or NC-6 strain tachyzoites were used as antigen (data not shown).

Cellular immune responses

γIFN production post-inoculation was detected in three of four cows from SN-NC6 group (cows nos. 257, 603, and 2005) and in two of four cows from SP-NC6 group (nos. 269 and 298). One cow from the seropositive control group (no. 220) had γIFN detectable levels (>0.1) during all the assays. In the seronegative control group, γIFN production was not detected.

Histopathological and immunohistochemical analysis

One SN-NC6 cow (no. 257) had a non-viable, severely autolyzed fetus at necropsy. One SP-NC6 cow (no. 242) aborted, and the fetus was not available for further examination. The remaining fetuses from SN-NC6 and SP-NC6 groups were viable at the moment of slaughter, and all of them had compatible *N. caninum* histopathological lesions. The main lesions found were multifocal meningoencephalitis, mononuclear pneumonitis, multifocal necrotizing myositis, multifocal placentitis with mineralization (Fig. 3), myocarditis, pericarditis, periportal hepatitis, and bronchiolitis. Tissue samples (12 of 31) from SN-NC6 group with *N. caninum* compatible lesions were positive by IHC. No IHC-positive samples were found in fetuses of seropositive cows inoculated with NC-6 strain (nos. 265, 269, and 298). Mild lesions were observed in fetuses of SPC group cows (nos. 220, 229, 241, and 299); however, no IHC-positive samples were found. Finally, fetuses of SNC group did not show histopathological lesions compatible with *N. caninum*

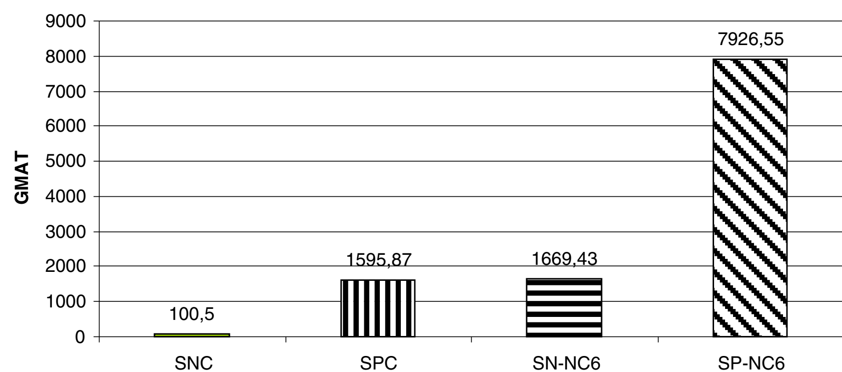

Fig. 2 Total geometric mean antibody titers (*GMAT*) for groups of bovines experimentally inoculated with NC-6 Argentina strain of *N. caninum* and controls. *SNC* uninfected negative control group, *SPC* uninfected positive control group, *SN-NC6* infected seronegative group, *SN-NC6* infected seropositive group

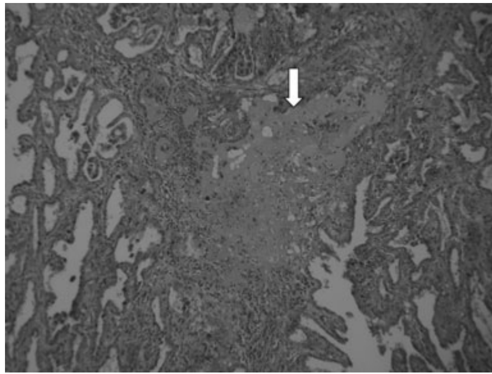

Fig. 3 Necrotic lesions in bovine placenta of a dam inoculated with NC-6 Argentina *N. caninum* tachyzoites (cow no. 989, SN-NC6 group). Extensive necrosis (*arrow*) of the fetal trophoblast and stromal necrosis of the maternal septa. H&E staining ×100 magnification

infection. The score of histopathological lesions observed in placenta and fetal tissues is shown in Table 1.

PCR and molecular characterization

PCR was positive for *N. caninum* DNA in tissue samples from three out of four fetuses of SN-NC6 group (heart from fetus 257, brain and heart from fetus 603, and brain from fetus 2005), two out of four fetuses from SP-NC6 group (brain from fetuses 269 and 298), and in placentas from cows no. 257, 603, 989 (group SN-NC6), and 298 (group SP-NC6). These samples were further analyzed by multilocus microsatellite analysis and *N. caninum* DNA extracted from all four bovine placentas had a microsatellite pattern identical to that observed for the *N. caninum* NC-6 Argentina strain, and the same result was obtained with heart from fetus 257 and brain from fetuses 603 (group SN-NC6) and 298 (group SP-NC6). Nested-PCR amplification and analysis of all ten microsatellite-containing sequences was achieved for DNA of *N. caninum* tachyzoites of NC-6 Argentina, NC-1 and NC-GER6 strains. Microsatellite sequences and GenBank accession numbers are displayed in Table 2. No *N. caninum* DNA was detected in cow brains.

Discussion

For experimental infection, we selected the *N. caninum* NC-6 Argentina isolate, which displayed a unique multilocus microsatellite pattern allowing its differentiation from other *N. caninum* isolates worldwide. By microsatellite analysis, we were able to provide evidence that the strain used for experimental inoculation crossed the placentas of both seronegative and seropositive pregnant cows. Although potentially possible, due to the high genetic diversity of *N. caninum*, it is unlikely that other field isolate infecting the seropositive animals in this study had displayed an identical microsatellite pattern as the NC6 Argentina isolate used for the inoculations. Besides, the animals used in this experiment derived from a

Table 1 Score of histopathological lesions observed in placenta and fetal tissues from cattle experimentally inoculated with NC6 Argentina *N. caninum* isolate at 65 days of gestation and slaughtered at 108±2 days of gestation

Group	Identification (cow number)	Placenta and fetal tissues						
		Cot	CNS	H	Lu	Kd	Li	Mu
SN-NC6	257	+++	++	+++	+++	−	−	+++
	603	+++	+	+++	++	−	−	+++
	989	++	++	+++	+++	++	+++	+++
	2005	++	−	++	++	−	−	−
SP-NC6	265	+++	++	++	++	−	−	+++
	269	+++	+	++	++	−	−	++
	298	++	++	++	++	+++	+++	+
SNC	608	−	−	−	−	−	−	−
	2071	−	−	−	−	−	−	−
SPC	220	+	−	+	+	−	−	−
	229	++	+	+	+	−	−	−
	241	+	−	+	+	−	−	−
	299	+	−	+	+	−	−	−

Cot placental cotyledon; *CNS* central nervous system; *H* heart; *Lu* lung; *Kd* kidney; *Li* liver; *Mu* striated muscle; *SN-NC6* cattle seronegative (IFAT titer <1:25) to *N. caninum* inoculated with NC-6 Argentina strain; *SP-NC6* seropositive (IFAT titer ≥1:100) cattle, naturally infected with *N. caninum*, inoculated with NC-6 Argentina strain; *SNC* seronegative (IFAT titer <1:25), non-inoculated control cattle; *SPC* seropositive (IFAT titer ≥1:100), non-inoculated control cattle, naturally infected with *N. caninum*

Table 2 *N. caninum* microsatellite alleles found in NC-6 Argentina strain, analyzed by sequencing or capillary electrophoresis, compared to alleles in *N. caninum* NC-1 and NC-GER6 strains

Strain	*N. caninum* microsatellite sequence and length[a] (bp)										Ref.
	MS1B $(AT)_n AC\ (AT)_n$	MS2[c] $(AT)_n TTGTATC\ (AT)_n GT\ (AT)_n$	MS3 (AT)	MS4 (AT) ACATTT (AT)	MS5 (TA)TGTA	MS6A (TA)	MS6B (AT)	MS10[b] $(ACT)_n(AGA)_n\ (TGA)_n$	MS12 (GT)	MS21 (TACA)	
NC-6 Argentina[b]	$(AT)_9AC\ (AT)_3$	$(AT)_6TTGTATC\ (AT)_{10}GT\ (AT)_2$	$(AT)_9$	$(AT)_{10}ACATTT\ (AT)_2$				$(ACT)_6(AGA)_{14}\ (TGA)_8$			This study
	(26 bp)	(45 bp)	(18 bp)	(30 bp)	(22 bp)	24 bp)	(22 bp)	(84 bp)	(32 bp)	(40 bp)	
NC-1[c]	$(AT)_9AC\ (AT)_3$	$(AT)_7TTGTATC\ (AT)_{10}GT\ (AT)_2$	$(AT)_{17}$	$(AT)_{12}ACATTT\ (AT)_2$	$(TA)_{12}TGTA$			$(ACT)_7(AGA)_{12}\ (TGA)_9$			Basso et al. (2009, 2010)
	(26 bp)	(47 bp)	(34 bp)[d]	(34 bp)	(28 bp)	(24 bp)	(34 bp)	(84 bp)	(32 bp)	(40 bp)	
NC-GER6[c]	$(AT)_8AC\ (AT)_3$	$(AT)_6TTGTATC\ (AT)_{10}GT\ (AT)_2$	$(AT)_{12}$	$(AT)_{11}ACATTT\ (AT)_2$	$(TA)_{10}TGTA$			$(ACT)_6(AGA)_{17}\ (TGA)_8$			Basso et al., (2009, 2010)
	(24 bp)	(45 bp)	(24 bp)	(32 bp)	(24 bp)	(26 bp)	(22 bp)	(93 bp)	(34 bp)	(40 bp)	

MS microsatellite, *bp* base pairs, *n* number of repeats of the sequence motif, *Ref.* reference

[a] The length of the microsatellite markers was calculated based on sequence data produced by the *N. caninum* Sequencing Group at the Sanger Institute for Nc-Liv *N. caninum*-strain, available at ftp://ftp.sanger.ac.uk/pub/pathogens/Neospora/caninum/NEOS.contigs.version1, and on sequences reported by Regidor Cerrillo et al. (2006) and Basso et al. (2009b)

[b] Sequence accession nos. MS1B-NC6 Argentina JN642712, MS2-NC6 Argentina JN642713, MS4-NC6 Argentina JN642714, and MS10-NC6 Argentina JN642715

[c] *N. caninum*-MS sequences from NC-1 and NC-GER6 strains were previously reported by Basso et al. (2009, 2010)

[d] MS3-NC-1 strain sequence was corrected in the original submission, accession no. EU872366 (Basso et al. 2009)

farm located 500 km away from the city where NC6 Argentina parasites were isolated from a dog about 10 years before (Basso et al. 2001). Thus, we could show that microsatellite typing is an exceptional tool to achieve the molecular characterization of *N. caninum* isolates and demonstrated its usefulness in experimental infections. This is the first report of experimental infection of cows with a *N. caninum* isolate from Argentina.

It was previously shown in several studies that the antibody titer can be used as indicator of parasite activity on the immune system, so that an increase of the titer would be related to parasite multiplication in the host, after infection or reactivation of a preexistent infection (Innes et al. 2005). In the present study, *N caninum* antibody titers were significantly higher in the inoculated groups, both in previously seronegative and seropositive animals. Most likely the infection and the subsequent multiplication of the NC-6 Argentina strain were the causes of this increase in specific antibodies. In the SP-NC6 group, the antibody titers raised earlier and higher than in the SN-NC6 group. This could be attributed to the immune memory response in the SP-NC6 animals because they were most likely chronically infected by *N. caninum* prior to experimental inoculation. In addition, the cellular immune response was triggered by NC-6 Argentina infection, both in seropositive and seronegative cows. One cow from the seropositive, but not inoculated control group (SPC group), had γIFN detectable levels during all the assays, probably as a natural response to a previous *N. caninum* infection. The cellular immune response produced in infected cattle was not effective in preventing transplacental transmission of *N. caninum* to fetuses. This could be due to the early age of gestation in which the inoculation was made. The gestational age at the time when infection occurs is an important factor and determines the potential impact of the infection on the fetus. Williams et al. (2000) demonstrated by intravenous inoculation of *N. caninum* during early gestation (10 weeks) that transmission to the fetus at that time had an efficiency of 83 %, but at 30 weeks the efficiency reached 100 %. However, a challenge early in pregnancy had more serious consequences for fetuses than a later challenge (Williams et al. 2000; Gondim et al. 2004). In this work, the consequences caused by intravenous inoculation of the NC-6 Argentina strain at 65 days of gestation were severe for fetuses from both seropositive and seronegative animals at the time of infection. Two cows, one from SP-NC6 and one from SN-NC6 groups, aborted or had a non-viable fetus at slaughter, respectively, and the remaining fetuses had several lesions in most tissues, with IHC demonstrable parasites in SN-NC6 group. It is not known if these fetuses would have been aborted in a later stage of gestation. In a study with the NC-1 strain, Macaldowie et al. (2004) intravenously inoculated pregnant cattle at 70 days of gestation and concluded that

early inoculation caused rapid fetal death and injury in placenta and fetal tissues associated with the presence of parasites. In a study of placental immunity to *N. caninum* in early gestation, Maley et al. (2006) concluded that the cellular immune response to infection at this time could protect the dam, but the same response could increase the damage produced in the placenta by the parasite and this would be detrimental for fetal survival. Although the fetus aborted by the cow in group SP-NC6 could not be recovered, it is probable that the abortion was produced by the pathogenic action of the NC-6 Argentina strain, based on the fact that this strain was the only one identified in the fetal tissues and the placentas of the other experimentally infected cows. NC-6 Argentina strain proved to be virulent in both seropositive (IFAT titers 1:400, 1:1,600, and 1:3,200) and seronegative cows (IFAT <1:25). This strain was able to cross the placenta of pregnant cows and was also virulent for the fetuses, evidenced by the lesions observed in the histopathological and IHC examination.

The multilocus microsatellite typing technique allowed us to characterize the *N. caninum* isolate used for the experimental inoculations and to differentiate it from other *Neospora* parasites that could have been present in the evaluated cows (SP-NC6 group), or which had potentially infected the animals later during the experiment. With this technique, we found evidence that the parasite strain used in the experimental inoculations was the one that infected the placenta and fetuses, not only of seronegative animals but also of animals that were chronically infected with *N. caninum* at the beginning of the study (seropositive animals), suggesting that the previous infection did not protect against the transplacental transmission of the new *N. caninum* strain. This was in contrast to experimental studies that were a vaccination with life *N. caninum*-protected against *N. caninum*-induced fetopathy (Williams et al. 2007).

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References

Al-Qassab S, Reichel MP, Ellis J (2010) A second generation multiplex PCR for typing strains of *Neospora caninum* using six DNA targets. Mol Cell Probes 24:20–26

Al-Qassab S, Reichel MP, Ivens A, Ellis JT (2009) Genetic diversity amongst isolates of *Neospora caninum*, and the development of a multiplex assay for the detection of distinct strains. Mol Cell Probes 23:132–139

Anderson ML, Andrianarivo AG, Conrad PA (2000) Neosporosis in cattle. Anim Reprod Sci 60–61:417–431

Anderson ML, Blanchard PC, Barr BC, Dubey JP, Hoffman RL, Conrad PA (1991) *Neospora* like protozoan infection as a major cause of abortion in California dairy cattle. J Am Vet Med Assoc 198:241–244

Barr BC, Rowe JD, Sverlow KW, BonDurant RH, Ardans AA, Oliver MN, Conrad PA (1994) Experimental reproduction of bovine fetal *Neospora* infection and death with a bovine *Neospora* isolate. J Vet Diagn Invest 6:207–215

Basso W, Schares S, Minke L, Bärwald A, Maksimov A, Peters M, Schulze C, Müller M, Conraths FJ, Schares G (2010) Microsatellite typing and avidity analysis suggest a common source of infection in herds with epidemic *Neospora caninum*-associated bovine abortion. Vet Parasitol 173:24–31

Basso W, Venturini L, Venturini MC, Hill DE, Kwok OCH, Shen SK, Dubey JP (2001) First isolation of *Neospora caninum* from the feces of a naturally infected dog. J Parasitol 87:612–618

Basso W, Herrmann DC, Conraths FJ, Pantchev N, Vrhovec MG, Schares G (2009a) First isolation of *Neospora caninum* from the faeces of a dog from Portugal. Vet Parasitol 159:162–166

Basso W, Schares S, Bärwald A, Herrmann DC, Conraths FJ, Pantchev N, Vrhovec MG, Schares G (2009b) Molecular comparison of *Neospora caninum* oocyst isolates from naturally infected dogs with cell culture-derived tachyzoites of the same isolates using nested polymerase chain reaction to amplify microsatellite markers. Vet Parasitol 160:43–50

Beck HP, Blake D, Darde ML, Felger I, Pedraza-Diaz S, Regidor-Cerrillo J, Gomez-Bautista M, Ortega-Mora LM, Putignani L, Shiels B, Tait A, Weir W (2009) Molecular approaches to diversity of populations of apicomplexan parasites. Int J Parasitol 39:175–189

Björkman C, Uggla A (1999) Serological diagnosis of *Neospora caninum* infection. Int J Parasitol 29:1497–1507

Campero CM, Anderson ML, Conosciuto G, Odriozola H, Bretschneider G, Poso MA (1998) *Neospora caninum*-associated abortion in a dairy herd in Argentina. Vet Record 143:228–229

Campero C, Moore D, Odeon A, Cipolla A, Odriozola E (2003) Aetiology of bovine abortion in Argentina. Vet Res Commun 27:359–369

Davison HC, Otter A, Trees AJ (1999) Estimation of vertical and horizontal transmission parameters of *Neospora caninum* infection in dairy cattle. Int J Parasitol 29:1683–1689

Dubey JP (1999) Neosporosis in cattle: biology and economic impact. J Am Vet Med Assoc 214:1160–1163

Dubey JP (2003a) Neosporosis in cattle. J Parasitol 89(Suppl):S42–S56

Dubey JP (2003b) Review of *Neospora caninum* and neosporosis in animals. Korean J Parasitol 60:1–16

Dubey JP, Hattel AL, Lindsay DS, Topper MJ (1988) Neonatal *Neospora caninum* infection in dogs: isolation of the causative agent and experimental transmission. J Am Vet Med Assoc 193:1259–1263

Dubey JP, Schares G (2006) Diagnosis of bovine neosporosis. Vet Parasitol 140:1–34

Gondim LF, McAllister MM, Pitt WC, Zemlicka DE (2004) Coyotes (*Canis latrans*) are definitive hosts of *Neospora caninum*. Int J Parasitol 34:159–161

Gottstein B, Hentrich R, Wyss B, Thür A, Busato A, Stärk KDC, Müller N (1998) Molecular and immunodiagnostic investigations on bovine neosporosis in Switzerland. Int J Parasitol 28:679–691

Hall CA, Reichel MP, Ellis JT (2005) Neospora Abortion in dairy cattle: diagnosis, mode of transmission and control. Vet Parasitol 128:231–241

Innes EA, Andrianarivo AG, Björkman C, Williams DJL, Conrad PA (2002) Immune responses to *Neospora caninum* and prospects for vaccination. Trends Parasitol 18:497–504

Innes EA, Wright S, Bartley P, Maley S, Macaldowie C, Esteban-Redondo I, Buxton D (2005) The host–parasite relationship in bovine neosporosis. Vet Immunol Immunopathol 108:29–36

Jenkins M, Baszler T, Björkman C, Schaes G, Williams D (2002) Diagnosis and seroepidemiology of *Neospora caninum*-associated bovine abortion. Int J Parasitol 32:631–636

Macaldowie C, Maley SW, Wright S, Bartley P, Esteban-Redondo I, Buxton D, Innes EA (2004) Placental pathology associated with

fetal death in cattle inoculated with *Neospora caninum* by two different routes in early pregnancy. J Comp Path 131:142–156

Maley SW, Buxton D, Macaldowie CN, Anderson IE, Wright SE, Bartley PM, Esteban-Redondo I, Hamilton CM, Storsety AK, Innes EA (2006) Characterization of the immune response in the placenta of cattle experimentally infected with *Neospora caninum* in early gestation. J Comp Path 135:130–141

Mc Allister M, Dubey JP, Lindsay DS, Jolley WR, Wills RA, Mc Guire AM (1998) Dogs are definitive hosts of *Neospora caninum*. J Parasitol 28:1473–1478

Moré G, Basso W, Bacigalupe D, Venturini MC, Venturini L (2008) Diagnosis of *Sarcocystis cruzi*, *Neospora caninum*, and *Toxoplasma gondii* infections in cattle. Parasitol Res 102:671–675

Moré G, Bacigalupe D, Basso W, Rambeaud M, Beltrame F, Ramirez B, Venturini MC, Venturini L (2009) Frequency of horizontal and vertical transmission for *Sarcocystis cruzi* and *Neospora caninum* in dairy cattle. Vet Parasitol 160:51–54

Paré J, Thurmond MC, Hietala SK (1996) Congenital *Neospora caninum* infection in dairy cattle and associated calfhood mortality. Can J Vet Res 60:133–139

Pedraza-Diaz S, Marugan-Hernandez V, Collantes-Fernandez E, Regidor-Cerrillo J, Rojo-Montejo S, Gomez-Bautista M, Ortega-Mora LM (2009) Microsatellite markers for the molecular characterization of *Neospora caninum*: application to clinical samples. Vet Parasitol 166:38–46

Regidor-Cerrillo J, Pedraza-Diaz S, Gomez-Bautista M, Ortega-Mora LM (2006) Multilocus microsatellite analysis reveals extensive genetic diversity in *Neospora caninum*. J Parasitol 92:517–524

Regidor-Cerrillo J, Gomez-Bautista M, Pereira-Bueno J, Aduriz G, Navarro-Lozano V, Risco-Castillo V, Fernandez-Garcia A, Pedraza-Diaz S, Ortega-Mora LM (2008) Isolation and genetic characterization of *Neospora caninum* from asymptomatic calves in Spain. Parasitology 135:1651–1659

Rojo-Montejo S, Collantes-Fernandez E, Regidor-Cerrillo J, Alvarez-Garcia G, Marugan-Hernandez V, Pedraza-Diaz S, Blanco-Murcia J, Prenafeta A, Ortega-Mora LM (2009) Isolation and characterization of a bovine isolate of *Neospora caninum* with low virulence. Vet Parasitol 159:7–16

SENASA Resolución N° 115 (1999) Plan Nacional de Control y Erradicación de la Brucelosis y Tuberculosis. Secretaría de Agricultura, Ganadería, Pesca y Alimentación, Argentina

Thilsted JP, Dubey JP (1989) Neosporosis-like abortion in a herd of dairy cattle. J Vet Diagn Invest 1:205–209

Thurmond MC, Hietala SK (1997) Effect of *Neospora caninum* infection on milk production in first-lactation dairy cows. J Am Vet Med Assoc 210:672–674

Thurmond MC, Hietala SK (1996) Culling associated with *Neospora caninum* infection in dairy cows. Am J Vet Res 57:1559–1562

Venturini MC, Venturini L, Bacigalupe D, Machuca M, Echaide I, Basso W, Unzaga JM, Di Lorenzo C, Guglielmone A, Jenkins MC, Dubey JP (1999) *Neospora caninum* infections in bovine fetuses and dairy cows with abortions in Argentina. Int J Parasitol 29:1705–1708

Yamage M, Flechtner O, Gottstein B (1996) *Neospora caninum*: specific oligonucleotide primers for the detection of brain "cyst" DNA of experimentally infected nude mice by the polymerase chain reaction (PCR). J Parasitol 82:272–279

Williams DJL, Guy CS, McGarry JM, Guy F, Tasker L, Smith RF, MacEachern K, Cripps PJ, Kelly DF, Trees AJ (2000) *Neospora caninum*-associated abortion in cattle: the time of experimentally induced parasitaemia during gestation determines foetal survival. Parasitology 121:347–358

Williams DJL, Guy CS, Smith RF, Ellis J, Björkman C, Reichel MP, Trees AJ (2007) Immunization of cattle with live tachyzoites of *Neospora caninum* confers protection against fetal death. Infect Immun 75:1343–1348