Veterinary Parasitology 187 (2012) 72–78

Contents lists available at SciVerse ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

Experimental inoculation of *Neospora caninum* in pregnant water buffalo

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ARTICLE INFO

Article history:
Received 16 August 2011
Received in revised form
13 December 2011
Accepted 21 December 2011

Keywords:
Neospora caninum
Fetal pathology
Water buffalo

ABSTRACT

The aim of this study was to characterize the pathogenesis of *Neospora caninum* in experimentally inoculated pregnant water buffalo (*Bubalus bubalis*). Twelve Mediterranean female water buffaloes ranging in age from 4 to 14 years old and seronegative to *N. caninum* by indirect fluorescent antibody test (IFAT) were involved. Ten females were intravenously inoculated with 10^8 tachyzoites of NC-1 strain at 70 ($n = 3$) or 90 ($n = 7$) days of pregnancy (dp). Two control animals were inoculated with placebo at 70 and 90 dp, respectively. Serum samples were obtained weekly following inoculation to the end of the experiment. Three animals inoculated at 70 dp were slaughtered at 28 days post inoculation (dpi), three animals inoculated at 90 dp were slaughtered at 28 dpi and the remaining four animals inoculated at 90 dp were slaughtered at 42 dpi. Fetal fluids from cavities and tissue samples were recovered for IFAT and histopathology, immunohistochemistry and PCR, respectively. Genomic DNA from fetal tissues was used for parasite DNA detection and microsatellite genotyping in order to confirm the NC-1 specific-infection. Dams developed specific antibodies one week after the inoculation and serological titers did not decrease significantly to the end of the experiment. No abortions were recorded during the experimental time; however, one fetus from a dam inoculated at 70 dp was not viable at necropsy. Specific antibodies were detected in only two fetuses from dams inoculated at 90 dp that were slaughtered at 42 dpi. No macroscopic changes in the placentas and organs of viable fetuses were observed. Nonsuppurative placentitis was a common microscopic observation in *Neospora*-inoculated specimens. Microscopic fetal lesions included nonsuppurative peribronchiolar interstitial pneumonia, epicarditis and myocarditis, interstitial nephritis, myositis and periportal hepatitis. Positive IHC results were obtained in two fetuses from dams inoculated at 70 dp and slaughtered at 28 dpi. *N. caninum* DNA was detected in placentas and fetuses from all inoculated animals. The pattern of amplified microsatellites from placental and fetal tissues resembled the NC-1 strain. Water buffaloes, like cattle, are susceptible to experimental inoculation with *N. caninum* at early pregnancy.

1. Introduction

Water buffaloes are better adapted to wet tropical and subtropical environments than bovines. Approximately 100,000 water buffaloes (*Bubalus bubalis*) are raised

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doi:10.1016/j.vetpar.2011.12.030

under intensive conditions in wetlands of the northeast of Argentina according to national records, (NEA) (Crudeli and Patiño, 2011). With a high impact on the regional economy, the buffalo industry is increasing in the NEA area; however causes of reproductive failure have not yet been characterized. Initial studies in Argentina detected *Neospora caninum*-seropositive individuals with increased seroprevalence in mature water buffaloes (Campero et al., 2007).

N. caninum is an Apicomplexan parasite that can infect many animal species including water buffalo. Neosporosis is a severe disease causing reproductive losses in cattle but little is known about the pathogenesis of *Neospora*-infection in water buffalo. *Neospora*-like tissue cysts have been found in two of four aborted water buffalo fetuses in Italy (Guarino et al., 2000) and the parasite has been also isolated from naturally infected water buffalo (Rodrigues et al., 2004). Occurrence of specific antibodies has been described in Brazil (Fujii et al., 2001; Rodrigues et al., 2005; Gennari et al., 2005; Gondim et al., 2007), Egypt (Dubey et al., 1998), India (Meenakshi Sandhu et al., 2007), Vietnam (Huong et al., 1998), and Italy (Guarino et al., 2000) with seroprevalence varying from 1.5% to 70.9%. Despite the high seropositivity and the evidence of vertical transmission (Chryssafidis et al., 2010), spontaneous abortion caused by *N. caninum* has not yet been described in water buffalo. Moreover, experimental vertical transmission or abortion by *N. caninum* has not been previously reported in pregnant water buffalo. The consequences of experimental inoculation with *N. caninum* in pregnant water buffalo are described in this study.

2. Materials and methods

2.1. Animals and experimental infection design

Twelve Mediterranean female water buffaloes (*B. bubalis*) aged 4–14 years were involved in the study. The females were seronegative by IFAT for *N. caninum* (reciprocal serum dilution lower than 100). They were also seronegative to *Brucella abortus* by buffered plate antigen (BPA) test (Angus and Barton, 1984). No measurable antibody titers to BHV-1 and BVDV-1 were detected by serum neutralization technique, using dilutions of serum (from 1:8 to endpoint titer) against a fixed dose of virus (100 $TCID_{50}$) (Rossi and Kiesel, 1971).

After estrus synchronization and impregnated by natural breeding, the animals were divided into four groups as follows: (A) three animals were inoculated at 70 days of pregnancy (dp) and slaughtered 28 days post inoculation (dpi); (B) three animals were inoculated at 90 dp and slaughtered 28 dpi; (C) four animals were inoculated at 90 dp and slaughtered 42 dpi; and (D) two control animals each one received mock inoculum at 70 and 90 dp were slaughtered at 28 dpi.

All the animal groups were fed on a natural pasture in one paddock and maintained under standard animal husbandry conditions. Clean water was available at all times. Pregnancy was confirmed by ultrasound 30 days after mating and weekly thereafter until slaughter. Rectal temperatures were recorded weekly.

All animals used in this study were handled in strict accordance with good animal practice and the conditions defined by the Animal Ethics Committee at the National Institute of Agrarian Technology, Argentina. All efforts were made to minimize suffering.

2.2. Culture of N. caninum tachyzoites

N. caninum tachyzoites of the NC-1 strain were harvested from VERO cell monolayer when 80% of them were infected. Tachyzoites were released by sequential passage of the cell monolayer through 21, 23 and 25 gauge needles. Parasites were washed with sterile phosphate buffer solution (PBS), counted with a hemocytometer and finally used either to make the antigen for IFAT or the live inoculum (Dubey et al., 1988).

2.3. Inoculums

Adjusting 1×10^8 protozoa as total dose per female, tachyzoites were diluted in 3 ml PBS and packed in 5 ml sterile syringes. Parasite inocula was transported in an insulated box at room temperature (RT) and administered intravenously to the females within 45 min after harvest from the tissue culture. An aliquot of the same parasites used for inoculation of animals was used to infect VERO cells. Parasite growth was observed after 5 days, confirming the parasite viability.

Similarly, two seronegative buffalo females (negative controls) were inoculated with a mock preparation from uninfected VERO cells in PBS (Dubey et al., 1988; Moore et al., 2011). VERO cells were treated in the same way as those that were infected with tachyzoites.

2.4. Clinical monitoring, necropsy procedures and samples

All animals were monitored daily until slaughtering. Rectal temperatures were recorded from the day prior to inoculation and for 10 days after inoculation.

Blood samples were collected from the jugular vein at 1-week intervals from week 0 (moment of the inoculation) to the end of the experiment. After separation, serum samples were stored at −20 °C until assessed by IFAT.

Following slaughter the fetuses were examined following standard fetal necropsy procedures. Fetal length was estimated using crown-rump measurement (Hubbert, 1972; Kirkbride, 1986). Samples of lung and abomasal content were cultured for aerobic and microaerophilic organism (Campero et al., 2003). The *Campylobacter* fetus culture and direct immunoflourescence were performed on abomasal fluid as mentioned (Campero et al., 2003). Samples of abomasal content were also cultured for *Tritrichomonas* fetus in liver broth medium (Campero et al., 1986). Fetal spleen samples collected at necropsy were processed for virus isolation. At the time of virus isolation, a 10% tissue homogenate was prepared and inoculated onto cultures of MDBK cells. After 4 blind passages inoculated cultures were tested for Bovine Viral Diarrhea Virus (BVDV) and Bovine Herpesvirus (BHV) antigens by a direct immunofloures-cence with a commercially available polyclonal antibody

(VMRD, USA) (Campero et al., 2003). Placentome, chorioallantoic membrane and several fetal tissue samples [central nervous system (CNS), heart, lung, liver and thoracic and abdominal fluids] were collected for DNA extraction avoiding cross-contamination between cases. At the same time, placentome, chorioallantoic membrane and fetal tissues (CNS, heart, lung, kidney, liver and muscle) were also placed in 10% formalin solution for histology. Fetal fluids were collected from specimens and stored at −20 °C until assessed by IFAT (Campero et al., 2003).

2.5. Indirect fluorescent antibody test (IFAT)

IFAT was used as previously described (Dubey et al., 1988) using a fluorescein isothiocyanate (FITC) labeled affinity-purified rabbit anti-bovine IgG antibody (Sigma, St. Louis, USA) (Fujii et al., 2001; Campero et al., 2007). *N. caninum* specific antibodies were measured using dilutions of serum or fetal fluids from 1:100 (Fujii et al., 2001; Campero et al., 2007) and 1:10 to endpoint titer, respectively. Positive and negative control sera were used. Slides were examined with an epifluorescence microscope (Olympus Bx 51, Olympus Inc., Tokio, Japan). Antibody titers were expressed as the reciprocal of the highest serum dilution that showed distinct whole parasite fluorescence (Campero et al., 2007).

2.6. Histopathological examination

After fixation, placental cotyledons and associated membranes, and blocks of fetal brain, heart, lung and liver were cut for processing to paraffin wax as described by Campero et al. (2003). Sections were cut from all blocks and stained with hematoxylin and eosin (HE). Based on severity, four categories of histologic lesions were established: (0) absence of lesions, (1) hemorrhage and less than 3 foci of light non-suppurative inflammatory infiltrate, (2) presence of 3 or more foci of non-suppurative inflammatory infiltrate, and (3) multifocal inflammatory infiltrate and necrosis.

2.7. Immunohistochemistry

Cases with moderate to severe histopathological lesions (categories 2 and 3) compatible with those produced by *N. caninum* were processed for IHC performed by Avidin Biotin Complex (Vector, Peroxidasa Elite ABC PK-601) technique for *N. caninum* (Lindsay and Dubey, 1989). Briefly, a second set of paraffin sections were mounted on positive-charged glass slides (Probe-On Plus; Fisher Scientific) and processed for IHC as previously described with an automated capillary action immunostainer (Micro-Probe TM; Fisher Scientific). Sections were dehydrated, enzymatically treated with 0.4% pepsin (Sigma Chemical Co., St. Louis, MO) for antigen retrieval, and incubated with 0.5% casein (Sigma Chemical Co., St. Louis, MO) to block nonspecific immunoglobulin binding. The primary antibody was anti-*N. caninum* hyperimmune polyclonal rabbit serum (kindly provided by Dr. M. Anderson from UC Davis, US) diluted 1:200. Immunostaining was visualized with amino-ethylcarbazol substrate (Dako Inc.), and sections were counterstained with Mayer's hematoxylin (Sigma Diagnostics, St. Louis, MO) and examined microscopically. Positive control tissue consisted of formalin-fixed brain tissue from mice experimentally inoculated with the NC-1 strain of *N. caninum*. Negative controls consisted of replacement of the primary antibody with a similar dilution of normal rabbit serum.

2.8. Nested PCR (nPCR)

DNA was isolated from tissue samples using a commercially available kit according to the manufacturer's recommendations (Dneasy Tissue Kit, QIAGEN Group, Germany). A PCR was used following protocols previously described (Buxton et al., 1998). Secondary amplification product was analyzed directly by 2% agarose gel electrophoresis and ethidium bromide fluorescence.

2.9. Microsatellite genotyping

PCR-positive DNA samples from placenta and fetal tissues were used for *Neospora* genotyping. A total of 9 previously described (Regidor-Cerrillo et al., 2006) microsatellite markers (MS4, MS5, MS6A, MS6B, MS7, MS8, MS10, MS12 and MS21) were amplified by nested-PCR using the primers and PCR conditions, and subsequently identified by allele size and sequencing as described by Pedraza-Díaz et al. (2009). DNA extracted from NC-1 isolate used in inoculations was also simultaneously genotyped.

2.10. Statistical analysis

Serum antibody responses (IFAT titers) were compared between groups using PROC-MIXED SAS for one-way repeated measures analysis of variance (ANOVA) with treatment as the grouping factor and time as the repeated measures factor (Littell et al., 1998). Data were log10-transformed before statistical analyses.

3. Results

Neither detrimental effects nor clinical signs (fever or abortions) were observed; however, one fetus from one inoculated dam in Group A was non-viable after slaughter. The averages of crown-rump length and their standard deviations were as follows: Group A: 13.7 ± 4.5 cm; Group B: 20.7 ± 1.1 cm; and Group C: 24.5 ± 2.8 cm. The crown-rump length for the two control fetuses inoculated at 70 and 90 dg were 17 and 15 cm, respectively. In contrast, the length of the dead fetus from Group A was 9 cm (Fig. 1).

The reciprocal antibody titers obtained by IFAT for animals in Groups A, B and C are shown in Table 1. Specific antibody titers to *N. caninum* developed in all females after the inoculation. IFAT-titers varied from 3200 to 51,200 having a significant increment one week after the inoculation ($P < 0.05$). The mean of the IFAT-titers did not decrease significantly in animals from Groups A, B and C by the time of slaughter ($P > 0.05$). Interestingly, the dam of the non viable fetus from Group A had the highest IFAT-titer (Table 1). Animals from Group D remained seronegative during the

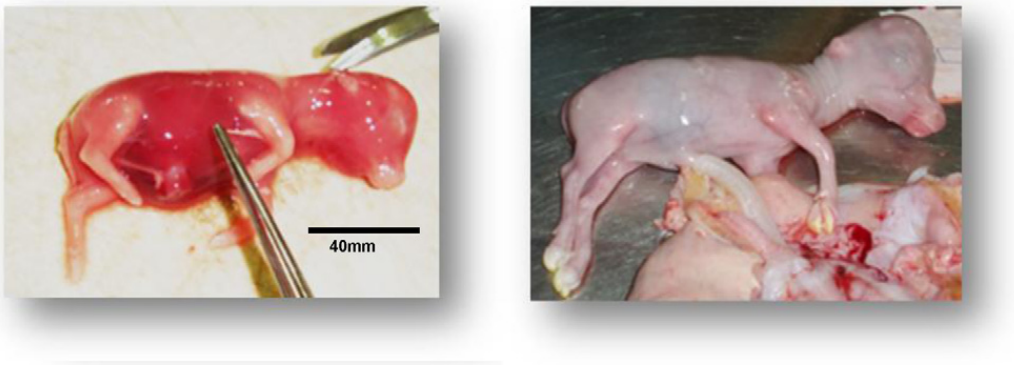

Fig. 1. Left; dead fetus #3 (length 9 cm) from Group A; right: viable fetus (length 15 cm) # 1 from Group D.

Table 1
N. caninum-antibody titers by IFAT in pregnant female water buffaloes from Group A inoculated at 70 dp and slaughtered 28 dpi; Group B inoculated at 90 dp and slaughtered 28 dpi; Group C inoculated at 90 dp and slaughtered 42 dpi. Animals from Group D remained without significant variations in their IFAT titers (data not shown).

Group	ID	0	7	14	21	28	35	42
A	1	<200	3200	1600	6400	800	NA	NA
	2	<200	12,800	12,800	200	800	NA	NA
	3[a]	<200	12,800	12,800	12,800	25,600	NA	NA
B	1	<200	6400	1600	6400	1600	NA	NA
	2	<200	12,800	12,800	25,600	6400	NA	NA
	3	<200	6400	3200	400	800	NA	NA
C	1	<200	6400	12,800	400	6400	6400	6400
	2	<200	51,200	51,200	25,600	6400	25,600	25,600
	3	<200	12,800	6400	6400	12,800	6400	6400
	4	<200	6400	6400	12,800	6400	6400	6400

ID: identification; NA: not available.
[a] Dam of the non viable fetus.

study (data not shown). Specific antibodies (≥1:10) were only detected in fluids of two fetuses from Group C.

Nonsuppurative placentitis was a frequent lesion in *Neospora*-infected specimens (9/10 specimens) (Table 2). Meningoencephalitis was characterized by gliosis and light non-suppurative inflammatory infiltration in the pia mater (4/10 specimens). Multiple necrotizing foci in the CNS were rarely observed (Fig. 2). Multiple mononuclear inflammatory foci were observed in different fetal tissues such as lung (peribronchiolar interstitial pneumonia) in 10/10 specimens (Fig. 3), heart (epicarditis and myocarditis) in 9/10 specimens, kidney (interstitial nephritis) (8/10 specimens), striated muscle (8/10 specimens) and liver (periportal hepatitis) in 8/10 specimens. No histopathological lesions were observed in the two control fetuses (data not shown).

N. caninum was identified by IHC in fetuses #1 and #3 from Group A. In fetus #1, tachyzoites were identified associated with focal necrosis and gliosis in the CNS. These parasitic stages were also observed in the dead fetus #3 in clusters in the heart, liver, lung, kidney, retro-ocular and striated muscles (Fig. 4).

N. caninum DNA was detected in 8 of 10 placentas and 9 of 10 fetuses from the inoculated animals (Table 2). Placenta and fetal tissues from the two control fetuses were negative by nPCR (data not shown). Complete or almost complete microsatellite profiles were also obtained from

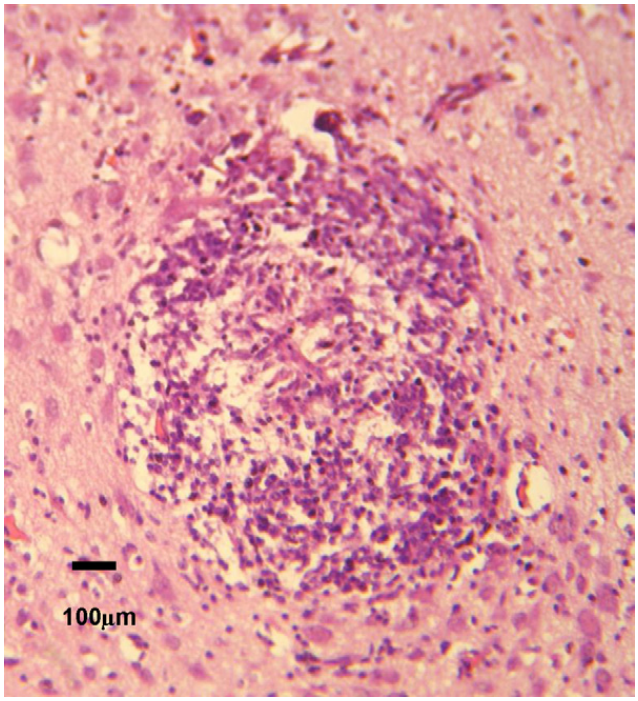

Fig. 2. Photomicrographs of focal necrosis surrounded by mononuclear inflammatory cells and glial cells in a section of the brain of water buffalo fetus #1 from Group A (H&E stain 200×).

Table 2
Score of histopathological lesions and results by nPCR recorded in placenta and fetal tissues from water buffaloes inoculated with *N. caninum* and slaughtered at different periods of gestation. Four categories of histologic lesions were established: (0) absence of lesions, (1) hemorrhage and less than 3 foci of light non-suppurative inflammatory infiltrate, (2) presence of 3 or more foci of non-suppurative inflammatory infiltrate, and (3) multifocal inflammatory infiltrate and necrosis.

Group	Fetal age	ID	Placenta and fetal specimens													
			P		CAM		CNS		H		Lu		Li		FF	
			H	nPCR	H	nPCR	H	nPCR	H	nPCR	H	nPCR	H	nPCR	H	nPCR
A	98	1	1	+	1	+	2	+	1	–	1	–	1	–	ND	–
		2	0	+	0	+	0	–	1	–	1	–	1	–	ND	–
		3	3	+	3	+	NA	NA	3	+	3	+	3	+	ND	NA
B	118	1	1	–	0	–	1	–	3	+	3	+	2	–	ND	–
		2	2	+	1	+	0	+	3	+	2	+	3	+	ND	+
		3	1	+	0	+	1	+	1	–	1	–	2	–	ND	–
C	132	1	2	+	1	+	0	+	2	–	3	+	0	–	ND	–
		2	2	+	1	+	0	+	0	+	1	+	0	–	ND	+
		3	1	+	1	+	0	+	2	+	3	+	3	+	ND	+
		4	1	–	1	–	1	–	1	+	1	–	0	+	ND	+

ID: identification; P: placentoma; CAM: chorioallantoic membrane; CNS: central nervous system; H: heart; Lu: lung; Li: liver; FF: fetal fluids; NA: not available; ND: not done.

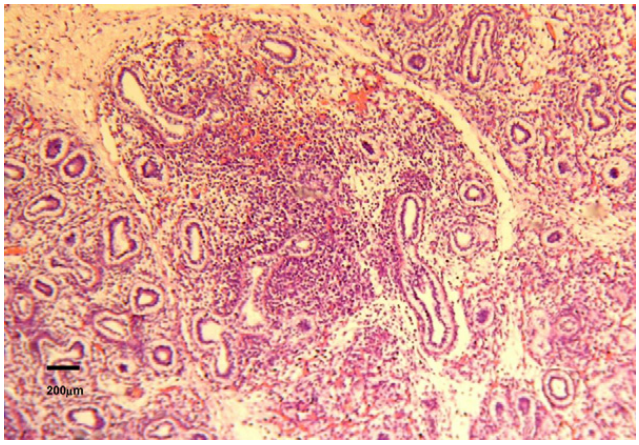

Fig. 3. Photomicrographs of focus of mononuclear inflammatory cells in sections of lung from of water buffalo fetus #1 from Group B. (H&E stain 100×).

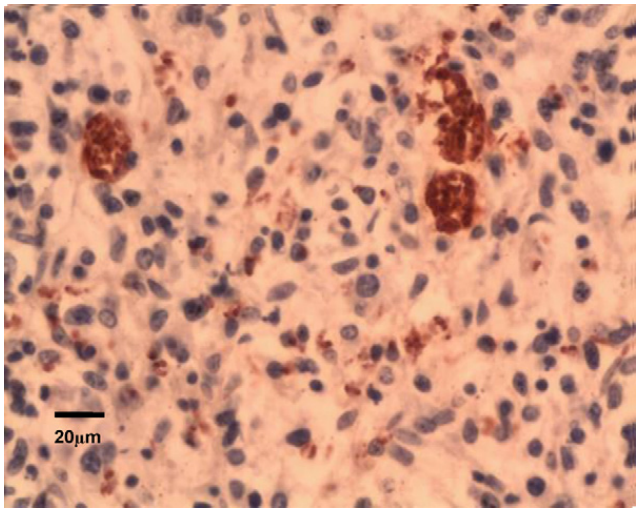

Fig. 4. Clusters of *N. caninum* tachyzoites detected by IHC in striated muscle in water buffalo fetus #3 from Group A (400×).

several placentas and the lung from the dead fetus #3 in Group 1 (Table 3). The microsatellite profiles were consistent with that determined for the NC-1 isolate used in inoculations, confirming that the source of placental and fetal tissue infection can be attributed to the experimental inoculations.

4. Discussion

The buffalo industry has a high impact on several regional economies including the NE of Argentina (Crudeli and Patiño, 2011). The causes of reproductive failure in buffaloes in Argentina have not been characterized; nevertheless, a high proportion of *Neospora*-seropositive water buffalo have been reported in four ranches (Campero et al., 2007). Although evidence of natural *Neospora* infections have been described (Rodrigues et al., 2004; Chryssafidis et al., 2010), spontaneous abortion caused by *N. caninum* has not yet been reported in water buffaloes. In this work we show that *N. caninum* can be transmitted vertically, it causes placental and fetal inflammatory lesions; and, eventually fetal death in water buffalo females after experimental inoculation during early gestation. The rise in specific antibodies following inoculation, the demonstration of the parasite in the fetus by IHC, PCR and the genetic characterization confirming the NC-1 profile in infected specimens indicate that the changes found were a direct result of the experimental *Neospora*-infection.

The antibody titers recorded in water buffaloes in this work (average 10,000) were higher and appeared earlier (one week after the experimental inoculation) than those reported in cattle using the same parasite strain, dose, route of administration and gestational stage at the time of inoculation (average of the reciprocal IFAT-titers: 142, two weeks after the inoculation) (Caspe et al., unpublished data). This suggests that water buffalo may respond with a more robust *Neospora*-specific antibody response than cattle, nevertheless the reason for this difference remains to be explained. In chronically *Neospora*-infected cattle, it was recently demonstrated

Table 3
Microsatellite profiles for the NC-1 isolate and placental and fetal tissues from inoculated animals.

Group	ID NC-1	Specimen	[a]MS4 GC-$(AT)_{12}$-ACATTT-$(AT)_2$-AC	MS5 CG-$(TA)_{12}$-TGTA-GG	MS6A GC-$(TA)_{12}$-AC	MS6B CC-$(AT)_{16}$-GT	MS7 ATAA-$(TA)_{16}$	MS8 AC-$(AT)_{16}$-GG	MS10 $(ACT)_7$-$(AGA)_{12}$-$(TGA)_9$	MS12 GC-$(GT)_{16}$-GC	MS21 TG-$(TACA)_3$-TACC-$(TACA)_6$-TT
A	1	Placenta	GC-$(AT)_{12}$-ACATTT-$(AT)_2$-AC	CG-$(TA)_{12}$-TGTA-GG	GC-$(TA)_{12}$-AC	CC-$(AT)_{16}$-GT	ATAA-$(TA)_{16}$	NA	$(ACT)_7$-$(AGA)_{12}$-$(TGA)_9$	GC-$(GT)_{16}$-GC	TG-$(TACA)_3$-TACC-$(TACA)_6$-TT
	3	Placenta	GC-$(AT)_{12}$-ACATTT-$(AT)_2$-AC	CG-$(TA)_{12}$-TGTA-GG	GC-$(TA)_{12}$-AC	CC-$(AT)_{16}$-GT	ATAA-$(TA)_{16}$	AC-$(AT)_{16}$-GG	$(ACT)_7$-$(AGA)_{12}$-$(TGA)_9$	GC-$(GT)_{16}$-GC	TG-$(TACA)_3$-TACC-$(TACA)_6$-TT
		Lung	GC-$(AT)_{12}$-ACATTT-$(AT)_2$-AC	CG-$(TA)_{12}$-TGTA-GG	GC-$(TA)_{12}$-AC	CC-$(AT)_{16}$-GT	ATAA-$(TA)_{16}$	AC-$(AT)_{16}$-GG	$(ACT)_7$-$(AGA)_{12}$-$(TGA)_9$	GC-$(GT)_{16}$-GC	TG-$(TACA)_3$-TACC-$(TACA)_6$-TT
B	2	Placenta	GC-$(AT)_{12}$-ACATTT-$(AT)_2$-AC	CG-$(TA)_{12}$-TGTA-GG	GC-$(TA)_{12}$-AC	CC-$(AT)_{16}$-GT	ATAA-$(TA)_{16}$	AC-$(AT)_{16}$-GG	$(ACT)_7$-$(AGA)_{12}$-$(TGA)_9$	GC-$(GT)_{16}$-GC	NA
C	1	Placenta	GC-$(AT)_{12}$-ACATTT-$(AT)_2$-AC	CG-$(TA)_{12}$-TGTA-GG	GC-$(TA)_{12}$-AC	CC-$(AT)_{16}$-GT	ATAA-$(TA)_{16}$	AC-$(AT)_{16}$-GG	$(ACT)_7$-$(AGA)_{12}$-$(TGA)_9$	NA	TG-$(TACA)_3$-TACC-$(TACA)_6$-TT
	2	Placenta	GC-$(AT)_{12}$-ACATTT-$(AT)_2$-AC	CG-$(TA)_{12}$-TGTA-GG	GC-$(TA)_{12}$-AC	CC-$(AT)_{16}$-GT	ATAA-$(TA)_{16}$	AC-$(AT)_{16}$-GG	$(ACT)_7$-$(AGA)_{12}$-$(TGA)_9$	GC-$(GT)_{16}$-GC	NA
	3	Placenta	GC-$(AT)_{12}$-ACATTT-$(AT)_2$-AC	NA	GC-$(TA)_{12}$-AC	CC-$(AT)_{16}$-GT	ATAA-$(TA)_{16}$	AC-$(AT)_{16}$-GG	$(ACT)_7$-$(AGA)_{12}$-$(TGA)_9$	GC-$(GT)_{16}$-GC	NA

ID: identification. NA: not amplified.
[a] Alleles for each microsatellite (MS) are shown by the repetitive motive sequences expected according to fragment size analysis.

that non-aborting pure-breed beef cattle showed significantly higher antibody levels throughout pregnancy than pure-breed dairy cattle (Santolaria et al., 2010). Even though an anti-bovine IgG can be used in the IFAT because water buffalo and cattle are related species; not only breed but also species differences may result in different humoral immune responses to *N. caninum*.

The early humoral immune response detected in 2 fetuses from dams inoculated at 90 days of gestation and slaughtered at 42 dpi is interesting. No information regarding the fetal immune competence of buffalo is available, nevertheless it could be speculated that it is similar to the bovine fetus, which is mature about 110 days of gestation (Osburn et al., 1982). The evaluation of *Neospora*-specific antibodies in fetal fluids is a quick, cheap and easy test to demonstrate infection by vertical transmission in abortions greater than 110 days; however according to our findings the test has a low sensitivity even used at 1:10 dilution in the water buffalo.

Cattle have been shown to be highly susceptible to experimental inoculation with *N. caninum* at the 10th week of gestation (Williams et al., 2000). Because gestation is longer in water buffaloes (315 days in Mediterranean breed) compared with cattle (282 days), in this study the experimental inoculation was performed at the 70 and 90 dp. Although the histopathological lesions and positive PCR results were found in fetuses inoculated either at 70 or 90 dp, the positive IHC results in several fetal tissues and the microsatellite genotype amplification from the lung in the nonviable fetus, suggests a higher fetal susceptibility to the experimental inoculation at 70 rather than 90 dp.

Histopathological findings described in this study were similar to those reported in cattle (Williams et al., 2000). Focal necrosis in the CNS of one fetus was present with mild meningoencephalitis in 4 of 10 fetuses from inoculated dams. In contrast, over 80% of fetuses from inoculated dams showed multiple mononuclear inflammatory foci in lung, heart, liver, kidney or muscles. The diagnosis of *Neospora*-bovine abortion is based on the histological observation of the CNS (Dubey et al., 2007). Our findings may suggest that several fetal tissues including placenta could be needed to observe microscopic lesions caused by *N. caninum* in water buffaloes.

The PCR applied on placenta and fetal tissues showed a high sensitivity, even in the absence of microscopic fetal lesions. In addition, the microsatellite genotyping of the parasite allowed identification that the *N. caninum* NC-1 isolate was the unique isolate implicated in infection. The lower sensitivity of IHC compared with PCR observed in this study agrees with previous results reported by Baszler et al. (1999). Although IHC may not be a reliable diagnostic test for *N. caninum*, it provides excellent information about the localization of the parasites in the fetal tissue as we showed in Fig. 4.

N. caninum was transmitted vertically causing lesions in placenta and fetuses of water buffalo females experimentally inoculated in early gestation. This work demonstrates the potential of *N. caninum* as an abortigenic agent in water buffaloes; however, more studies are necessary to clarify

the role of *Neospora caninum* as cause of spontaneous abortion in this species.

Acknowledgments

This project was funded by two Research Grants from the Agencia Nacional de Promoción Científica y Tecnológica, PICT 2412, and from INTA, AESA 203971, Argentina. Authors thank Dr. Mark Anderson from U.C. Davis, USA for critical review and helpful comments.

References

Angus, R., Barton, C., 1984. The production and evaluation of a buffered plate antigen for use in the presumptive test for brucellosis. Dev. Biol. Stand. 56, 349–356.

Baszler, T.V., Gay, L.J.C., Long, M.T., Mathison, B.A., 1999. Detection by PCR of *Neospora caninum* in fetal tissues from spontaneous bovine abortions. J. Clin. Microbiol. 37, 4059–4064.

Buxton, D., Maley, S.W., Wright, S., Thomson, K.M., Rae, A.G., Innes, E.A., 1998. The pathogenesis of experimental neosporosis in pregnant sheep. J. Comp. Pathol. 118, 267–279.

Campero, C., Catena, M., Medina, D., 1986. Caldo infusión hígado para el cultivo de *Tritrichomonas foetus*. Vet. Arg. 3, 80–81.

Campero, C.M., Moore, D.P., Odeón, A.C., Cipolla, A.L., Odriozola, E., 2003. Aetiology of bovine abortion in Argentina. Vet. Res. Commun. 27, 359–369.

Campero, C.M., Pérez, A., Moore, D.P., Crudeli, G., Benitez, D., Draghi, M.G., Cano, D., Konrad, J.L., Odeón, A., 2007. Occurrence of antibodies against *Neospora caninum* in water buffaloes (*Bubalus bubalis*) on four ranches in Corrientes province. Argentina Vet. Parasitol. 150, 155–158.

Chryssafidis, A.L., Soares, R.M., Rodrigues, A.A.R., Carvalho, N.A.T., Gennari, S.M., 2010. Evidence of congenital transmission of *Neospora caninum* in naturally infected water buffalo (*Bubalus bubalis*) fetus from Brazil. Parasitol. Res., 741–743.

Crudeli, G.A., Patiño, M.E., 2011. Produção de Búfalos de Leite. Cap: 1. In: Origen do Búfalo e Principais Raças. Editora FEPAF, Corrientes, Argentina, p. 40.

Dubey, J.P., Hattel, A.L., Lindsay, D.S., Topper, M.J., 1988. Neonatal *Neospora caninum* infection in dogs: isolation of the causative agent and experimental transmission. J. Am. Vet. Med. Assoc. 193, 1259–1263.

Dubey, J.P., Romand, S., Hilali, M., Kwok, O.C.H., Thullies, P., 1998. Seroprevalence of antibodies to *Neospora caninum* and *Toxoplasma gondii* in water buffaloes (*Bubalus bubalis*) from Egypt. Int. J. Parasitol. 28, 527–529.

Dubey, J.P., Schares, G., Ortega-Mora, L.M., 2007. Epidemiology and control of neosporosis and *Neospora caninum*. Clin. Microbiol. Rev. 20, 323–367.

Fujii, T.U., Kasai, N., Nishi, S.M., Dubey, J.P., Gennari, S.M., 2001. Seroprevalence of *Neospora caninum* in female water buffaloes (*Bubalus bubalis*) from the southeastern region of Brazil. Vet. Parasitol. 99, 331–334.

Gennari, S.M., Rodrigues, A.A.R., Viana, R.B., Cardoso, E.C., 2005. Occurrence of anti-*Neospora caninum* antibodies in water buffaloes (*Bubalus bubalis*) from the Northern region of Brazil. Vet. Parasitol. 134, 169–171.

Gondim, L.F.P., Pinheiro, A.M., Almeida, M.A.O., 2007. Frequência de anticorpos anti-*Neospora caninum* em búfalos (*Bubalus bubalis*) criados no Estado da Bahia. Rev. Bras. Saúde Prod. An. 8 (2), 92–96.

Guarino, A., Fusco, G., Savini, G., Di Francesco, G., Cringoli, G., 2000. Neosporosis in water buffalo (*Bubalus bubalis*) in southern Italy. Vet. Parasitol. 91, 15–21.

Hubbert, W.T., 1972. Committee on bovine reproductive nomenclature. Recommendations for standardizing bovine reproductive terms. Cornell Vet. 62, 216–237.

Huong, L.T.T., Ljungstrom, B.L., Uggla, A., Bjorkman, C., 1998. Prevalence of antibodies to *Neospora caninum* and *Toxoplasma gondii* in cattle and water buffaloes in southern Vietnam. Vet. Parasitol. 75, 53–57.

Kirkbride, C.A., 1986. Examination of bovine and ovine fetuses. Veterinary clinics of North American. Food Anim. Pract. 2, 61–83.

Lindsay, D.S., Dubey, J.P., 1989. Immunohistochemical diagnosis of *Neospora caninum* in tissue sections. Am. J. Vet. Res. 50, 1981–1983.

Littell, R.C., Henry, P.R., Ammerman, C.B., 1998. Statistical analysis of repeated measures data using SAS procedures. J. Anim. Sci. 76, 1216–1231.

Meenakshi Sandhu, K.S., Ball, M.S., Kumar, H., Sharma, S., Sidhu, P.K., Sreekumar, C., Dubey, J.P., 2007. Seroprevalence of *Neospora caninum* antibodies in cattle and water buffaloes in India. J. Parasitol. 93, 1374–1377.

Moore, D.P., Echaide, I., Verna, A.E., Leunda, M.R., Cano, A., Pereyra, S., Zamorano, P.I., Odeón, A.C., Campero, C.M., 2011. Immune response to *Neospora caninum* native antigens formulated with immune stimulating complexes in calves. Vet. Parasitol. 175, 245–251.

Osburn, B.I., Maclachlan, N.J., Terrell, T.G., 1982. Ontogeny of the immune system. J. Am. Vet. Med. Assoc. 181, 1049–1052.

Pedraza-Díaz, S., Marugán-Hernández, V., Collantes-Fernández, E., Regidor-Cerrillo, J., Rojo-Montejo, S., Gómez-Bautista, M., Ortega-Mora, L.M., 2009. Microsatellite markers for the molecular characterization of *Neospora caninum*: application to clinical samples. Vet. Parasitol. 166, 38–46.

Regidor-Cerrillo, J., Pedraza-Díaz, S., Gómez-Bautista, M., Ortega-Mora, L.M., 2006. Multilocus microsatellite analysis reveals extensive genetic diversity in *Neospora caninum*. J. Parasitol. 92, 517–524.

Rodrigues, A.A.R., Gennari, S.M., Aguiar, D.M., Sreekumar, C., Hill, D.E., Miska, K., Vianna, M.C.B., Dubey, J.P., 2004. Shedding of *Neospora caninum* oocysts by dogs fed tissues from naturally infected water buffaloes (*Bubalus bubalis*) from Brazil. Vet. Parasitol. 124, 139–150.

Rodrigues, A.A.R., Gennari, S.M., Paula, V.S.O., Aguiar, D.M., Fujii, T.U., Starke-Buzetti, W., Machado, R.Z., Dubey, J.P., 2005. Serological response to *Neospora caninum* in experimentally and naturally infected water buffaloes (*Bubalis bubalis*). Vet. Parasitol. 129, 21–24.

Rossi, C.R., Kiesel, G.K., 1971. Microtiter test for detecting antibody in bovine serum to parainfluenza-3 virus, infectious rhinotracheitis virus and bovine viral diarrhea virus. Appl. Microbiol. 22, 32–36.

Santolaria, P., Almería, S., Martínez-Bello, D., Nogareda, C., Mezo, M., Gonzalez-Warleta, M., Castro-Hermida, J.A., Pabón, M., Yániz, J.L., López-Gatius, F., 2010. Different humoral mechanisms against *Neospora caninum* infection in purebreed and crossbreed beef/dairy cattle pregnancies. Vet Parasitol. 178, 70–76.

Williams, D.J.L., Guy, G.S., Mcgarry, J.W., Guy, F., Tasker, L., Smith, R.F., Maceachenr, K., Cripps, P.J., Kelly, D.F., Trees, A.J., 2000. *Neospora caninum* – associated abortion in cattle: the time of experimentally – induced parasitaemia during gestation determines foetal survival. Parasitology 121, 347–358.