Experimental Parasitology 155 (2015) 8–12

Contents lists available at ScienceDirect

Experimental Parasitology

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

Full length article

# Isolation and molecular characterization of a new *Neospora caninum* isolate from cattle in Argentina

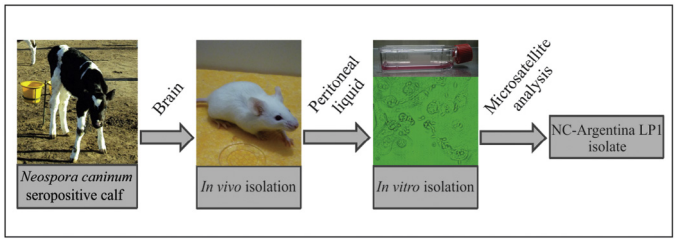
L.M. Campero [a,b], M.C. Venturini [b,*], D.P. Moore [a], L. Massola [c], H. Lagomarsino [d], B. García [c], D. Bacigalupe [b], M. Rambeaud [a,b], L. Pardini [b,e], M.R. Leunda [c], G. Schares [f], C.M. Campero [c]

[a] National Scientific and Technical Research Council (CONICET), Buenos Aires CP C1033AAJ, Argentina
[b] Immunoparasitology Laboratory, School of Veterinary Sciences, National University of La Plata, 60 and 118, La Plata 1900, Argentina
[c] National Institute of Agricultural Technology (INTA), Balcarce, 7620 Argentina
[d] Private Veterinary Practitioner
[e] Bunge and Born Foundation, Buenos Aires, Argentina
[f] Federal Research Institute for Animal Health, Institute of Epidemiology, Friedrich-Loeffler-Institute, Südufer 10, Greifswald, Insel Riems 17493, Germany

## HIGHLIGHTS

- Calves from a dairy farm with enzootic neosporosis were used for isolation.
- *In vivo* and *in vitro* isolation assays were performed.
- A new *Neospora caninum* isolate from an asymptomatic calf was achieved.
- Multilocus-microsatellite analysis was performed to characterize the isolate.
- We report the first bovine isolate of *N. caninum* in Argentina.

## GRAPHICAL ABSTRACT




## ARTICLE INFO

*Article history:*
Received 18 January 2015
Received in revised form 24 March 2015
Accepted 19 April 2015
Available online 22 April 2015

*Keywords:*
*Neospora caninum*
Isolate
Dairy asymptomatic calf
Argentina
Multilocus-microsatellite analysis

## ABSTRACT

*Neospora caninum* is one of the most important causes of bovine abortion, but isolation of live parasites from infected tissue is difficult. The aims of the present study were to obtain new isolates of *N. caninum* from congenitally infected asymptomatic newborn cattle in Argentina and to perform characterization by multilocus-microsatellite analysis. Five clinically normal born calves, with demonstrable *N. caninum* antibodies in precolostrum serum by indirect fluorescent antibody test, were euthanized and their brain samples were processed for histopathological, immunohistochemical, polymerase chain reaction (PCR) analysis, and for bioassay in γ-interferon knockout (GKO) mice. Although *N. caninum* DNA was detected in brain from all the calves by PCR, viable *N. caninum* was isolated in GKO mice from only one calf. *Neospora caninum* tachyzoites of this Argentinean isolate, designated NC-Argentina LP1, were propagated in *VERO* cell cultures seeded with tachyzoites from the infected GKO mice tissues. Multilocus-microsatellite typing on DNA derived from cell cultured tachyzoites revealed a unique genetic pattern, different from reported isolates. This is the first bovine isolation and genetic characterization of *N. caninum* in Argentina.


* Corresponding author. Fax: +54 221 4257980.
*E-mail address:* cventuri@fcv.unlp.edu.ar, cventuri9@gmail.com (M.C. Venturini).

http://dx.doi.org/10.1016/j.exppara.2015.04.009
0014-4894/

## 1. Introduction

*Neospora caninum* is a protozoan parasite with a worldwide distribution, considered a major cause of abortion in cattle (Dubey et al., 2007). *Neospora caninum* is one of the most efficiently transplacentally transmitted parasites among all known microbes in cattle. The efficiency of vertical transmission is important for the persistence of *N. caninum* within herds and can result in abortion, weak and underweight calves, or as in most cases, asymptomatic congenitally infected newborns (Dubey et al., 2007). In Argentina, cattle industry has socioeconomic importance and neosporosis has been reported as responsible for reproductive failure and important economic loss (Campero et al., 2003). The country has 54 million head of cattle: 3.3 million dairy and 50.7 million beef cattle. Economic losses due to *Neospora*-infections in the Humid Pampa of Argentina have been recently estimated at approximately US$44 million and US$13 million in dairy and beef cattle, respectively (Moore et al., 2013).

Isolation of *N. caninum* is difficult and the technique often ends in unsuccessful results. In addition, there is a limited number of isolates worldwide, most of them obtained from symptomatic animals (Dubey and Schares, 2006). New isolates from asymptomatic calves are important since they could display a natural reduced virulence, and, therefore, be useful in immunoprophylaxis studies (Regidor-Cerrillo et al., 2008). Even though bovine neosporosis has been reported in many South American countries, there are only records of bovine isolates in Brazil (Locatelli-Dittrich et al., 2004; Moore, 2005). Two *N. caninum* isolates were obtained in Argentina, one from feces of a naturally infected dog (Basso et al., 2001) and the other one from an axis deer fawn (Basso et al., 2014). Despite many attempts to obtain viable *N. caninum* isolates from cattle, this has not yet been achieved in Argentina (Venturini et al., 2000).

Multilocus-microsatellite analysis has been proven to be a valuable tool to study genetic diversity, to identify infection sources in molecular epidemiological studies, and to determine the sources of the isolates circulating in a delimited region (Basso et al., 2009). Microsatellites are highly variable loci which consist of tandemly repeated units of 1–6 base pair (bp) length, present in the genome of eukaryotic and prokaryotic organisms. The gain and loss of single repeat units in these loci cause polymorphisms in these sequences which can be used for genotyping individual organisms. Multilocus-microsatellite typing can be achieved either by sequencing or by assessing the length of amplified microsatellite-containing fragments by capillary electrophoresis, using fluorescent-labeled primers (Basso et al., 2009).

The aims of the work were to obtain new isolates of *N. caninum* from congenitally infected asymptomatic newborn cattle in Argentina and to perform characterization by multilocus-microsatellite analysis.

## 2. Materials and methods

### 2.1. Herd, animals and sampling

Animals came from a dairy herd (790 Holstein × Jersey cattle) located in Córdoba province, Argentina. The herd was free of brucellosis and tuberculosis with a seroprevalence of 21% for *N. caninum*. Precolostral blood samples from 47 calves (25 females and 22 males) were obtained by jugular vein puncture, and their respective dams were sampled 24–48 hours postpartum by coccygeal vein puncture. Sera were stored at –20 °C for further serological analysis. Five 7-day-old calves with the highest antibody levels were euthanized for isolation of viable *N. caninum*. During necropsy, half of the brain from each calf was aseptically removed, kept at 4 °C, and sent to Immunoparasitology Laboratory, School of Veterinary Sciences, La Plata. Simultaneously, formalin (10%) fixed and refrigerated calf tissues were processed for routine histological procedures and PCR studies.

### 2.2. Study on calves

#### 2.2.1. Indirect fluorescence antibody test

Indirect fluorescent antibody test (IFAT) was performed as described previously by Dubey et al. (1988) using a fluorescein isothiocyanate (FITC) labeled affinity-purified rabbit anti-bovine conjugate (Sigma-Aldrich, St. Louis, USA). *Neospora caninum* specific antibodies were assayed using dilutions from 1:25 to endpoint titer. Slides were examined with an epifluorescence microscope (Leica). Antibody titers were expressed as the reciprocal of the highest serum dilution that showed distinct whole parasite fluorescence (Paré et al., 1995).

#### 2.2.2. Histopathology and immunohistochemistry analysis

Brain and heart samples collected from the five calves were fixed in buffered 10% formalin, embedded in paraffin, sectioned, mounted, and stained with hematoxylin and eosin (H/E) (Campero et al., 2003). Tissues were processed for immunohistochemistry (IHC) for *N. caninum* presence by Avidin Biotin Complex using a commercial kit (ABC Elite ABC Peroxidase Complex Vector PK-601, Vector Laboratories, Burlingame CA, USA). Briefly, a second set of paraffin sections were mounted on positive-charged glass slides (Probe-On Plus; Fisher Scientific) and processed with an automated capillary action immunostainer (Micro-Probe TM; Fisher Scientific). The primary antibody was anti-*N. caninum* hyper-immune 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. Proper positive and negative controls were used (Campero et al., 1998).

#### 2.2.3. PCR assay

DNA was extracted using a commercial kit (Wizard® Genomic DNA Purification Kit, Promega) according to the manufacturer's protocol. A simple PCR was performed using the pair of primers Np6+/ Np21+, amplifying a 328 bp fragment (Müller et al., 1996). DNA from *N. caninum* NC-1 isolate was used as positive control and DNA from *VERO* cells as negative control. PCR results were analyzed by 2% agarose gel electrophoresis stained with SYBR safe (Invitrogen, USA) and compared to a standard molecular weight marker of 100 bp ladder.

### 2.3. Bioassay in mice

#### 2.3.1. Inoculum preparation

Half of the brains from each of the 5 *N. caninum* seropositive calves were processed individually. The material was homogenized with a blender in 20 ml of sterile physiological solution containing 0.5% (v/w) trypsin (Gibco®) and incubated 30 minutes at 37 °C. The homogenate was washed three times with 20 ml of phosphate buffered saline solution (PBS), and centrifuged at 600× *g* for 10 min. Supernatant was discarded and the pellet was resuspended in 5 ml of sterile physiological solution containing 2% antibiotic-antimycotic solution (1000 IU/ml penicillin G and 100 µg/ml of streptomycin) (Gibco® Antibiotic-Antimycotic).

#### 2.3.2. Immunodiagnostic tests and PCR

Twenty γ-interferon knockout mice (C.129S7 (B6)-Ifng$^{tm1Ts}$/], The Jackson Laboratory) were used for the bioassay. A total of 4 mice were subcutaneously (SC) inoculated, with 0.5 ml of each brain calf homogenate processed as detailed in section 2.3.1. Twenty six days post inoculation (dpi), mice were bled and sera were stored at –20 °C

for antibody detection against *N. caninum* by IFAT. Mice were sacrificed and organs were kept for HP, IHC, and PCR studies. Peritoneal fluids and brains were obtained and further passages of the brains (0.5 ml of PBS containing 2% antibiotic-antimycotic solution) were performed in mice.

IFAT on mice was performed as previously described, but goat anti-mouse conjugate (Sigma-Aldrich, St. Louis, USA) was used. *Neospora caninum* specific antibodies were measured using dilutions from 1:25 to endpoint titer. Histopathology and immunohistochemistry analysis were achieved as described in section *2.2.2*, with several organs collected: brain, heart, lungs, liver, spleen, kidneys, striated muscle, and tongue. Same specimens from infected GKO mice, cell cultures, and peritoneal washing ($2\times10^6$ tachyzoites) were assessed for PCR.

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 School of Veterinary Sciences, La Plata, Argentina. All efforts were made to minimize suffering.

### 2.4. In vitro *isolation assay*

The peritoneal cavity from inoculated mice was flushed with 3 ml sterile physiological solution. Approximately 1 ml of the recovered liquid was used to infect a 24 hour *VERO* cell monolayer maintained with RPMI 1640, 1000 IU/ml penicillin and 100 mg/ml streptomycin supplemented with 10% fetal bovine serum, in 25 $cm^2$ flasks. After 4 hours incubation at 37 °C, cell culture medium was changed. Flasks were incubated at 37 °C with 5% $CO_2$. Cell cultures were daily examined with an inverted microscope (Nikon).

### 2.5. *Microsatellite genotyping*

PCR-positive DNA samples from brain, liver, lungs and heart from seropositive mice and from $2\times10^8$ cell culture derived tachyzoites were used for *Neospora* genotyping by multilocus-microsatellite typing (MLST) for 10 microsatellites: MS1B, MS2, MS3, MS5, MS6A, MS6B, MS7, MS10, MS12 and MS21 of *N. caninum* (Regidor-Cerrillo et al., 2006). Microsatellites MS2 and MS10 were amplified and sequenced using primers and protocols described previously (Basso et al., 2009). The microsatellites, MS1B, MS3, MS5, MS6A, MS6B, MS7, MS12 and MS21, were analyzed by nested-PCR and fragment lengths determined by capillary electrophoresis.

### 2.6. *Nucleotide sequence accession numbers*

The nucleotide sequences of the MS2 and MS10 microsatellites analyzed in this study have been deposited in the GenBank database under accession numbers of KJ700414 and KJ700413, respectively.

## 3. Results

From the 47 calf-dam pairs tested, 27.6 % (13/47) of calves and 29.7% (14/47) of dams were seropositive. Precolostral titers from the five euthanized calves were 5–20 times higher compared to their respective dam's titer. Histopathological findings revealed mixed focal meningoencephalitis and epicarditis in 3 of 5 calves (Fig. 1), but *N. caninum* was not detected by IHC. *Neospora caninum*-DNA was detected by PCR in brains from the 5 calves.

One GKO mouse (M1) inoculated with brain of 1 of the 5 seropositive calves (IFAT 1:5120) became ill (ruffled fur, weakness and lethargy), tested seropositive (IFAT 1:400) at 26 dpi and was sacrificed. Brain from M1 was inoculated into 2 mice, M2 and M3. At 35 dpi, M2 and M3 were ill, became seropositive, and were sacrificed. A pool of brains from both mice was inoculated into mouse M4 that died 41 dpi. *Neospora caninum* DNA was identified by PCR

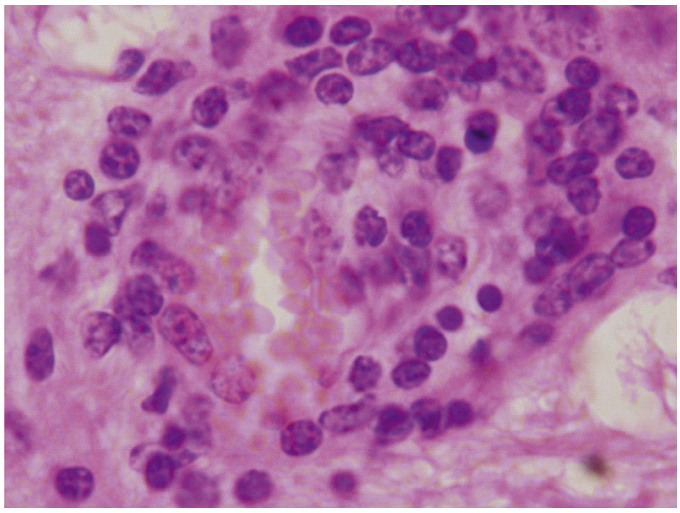


**Fig. 1.** Mild perivascular mononuclear cell inflammatory reaction in brain from a calf stained with H&E (×40).

in all infected mice tissues. Histopathological results on mice revealed multiple hemorrhages in brain, focal encephalitis with polymorphonuclear cells and mononuclear infiltrate and severe proliferative meningitis, severe and diffuse necrotizing myocarditis with mononuclear cells infiltration, interstitial pneumonitis characterized by mononuclear cells and multifocal periportal hepatitis. Lesions were associated with *N. caninum* tachyzoites identified by IHC (Fig. 2).

Peritoneal fluid with $0.5\times10^6$ total tachyzoites from M4 was used to inoculate a fresh monolayer *VERO* cell culture. Tachyzoites were observed 4 days post-inoculation in the flask. At day 14 after infection, more than 60% of the cell layer was disrupted and required subculture. Proliferation of the new isolate was slower (~30% cell disruption at 7 dpi) than the NC-1 isolate (~80% cell disruption), simultaneously kept in culture, with the same passage numbers.

Amplification of the 10 microsatellite-containing sequences and analysis by capillary electrophoresis and/or sequencing was achieved for *N. caninum*-PCR positive samples. Results are summarized in Table 1. Sequences of MS2 and MS10 were different from those of existing *N. caninum* isolates, i.e. sequences showed differences in

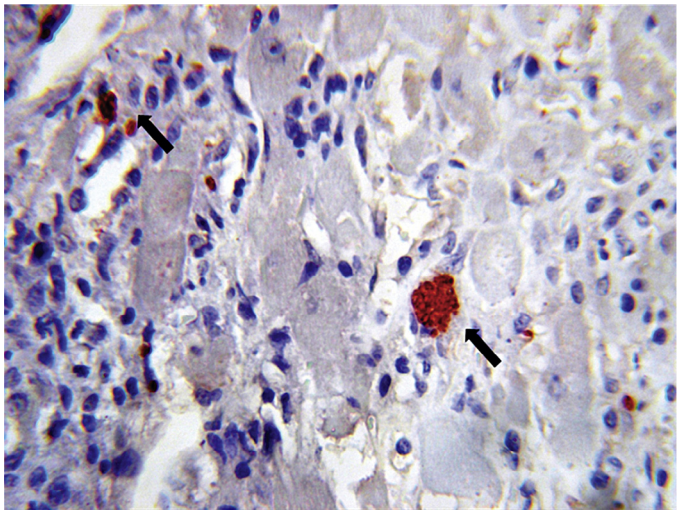


**Fig. 2.** Microphotography of myocardium section of infected mouse marked by IHC showing a cluster and isolated tachyzoites (arrows) and interstitial inflammation (ABC, ×40).

**Table 1**
*Neospora caninum* microsatellite alleles analyzed by capillary electrophoresis or sequencing for the new isolate.

| *N. caninum* MS | Microsatellite length (bp) (MS1B, 3, 5, 6A, 6B, 7, 12 and 21)[a] or sequence (MS2 and 10) Repeated sequence motif[a] (5– 3) | MS length (bp)[a] |
|---|---|---|
| MS 1B | (AT)AC(AT) | 26 |
| MS2 | (AT)*n*-TTGTATC-(AT)*n*-GT(AT)*n* | 6-10-2 |
| MS3 | (AT) | 24 |
| MS5 | (TA)TGTA | 32 |
| MS6A | (TA) | 32 |
| MS6B | (AT) | 26 |
| MS7 | (TA) | 20 |
| MS10 | (ACT)*n*-(AGA)*n*-(TGA)*n* | 6-15-9 |
| MS12 | (GT) | 32 |
| MS21 | (TACA) | 40 |

MS: microsatellite; bp: base pairs.
[a] The length of the amplification products and microsatellites was calculated using the Lasergene 7.0 Software (DNA Star Inc., Madison, USA) for sequences of the Nc-Liv *N. caninum* strain based on data produced by the Neospora caninum Sequencing Group at the Sanger Institute, available at ftp://ftp.sanger.ac.uk/pub/pathogens/Neospora/caninum/NEOS.contigs.version1.

the number of single repeat units relative to numbers reported for other isolates, thus confirming the unique multilocus genotype of this isolate, distinct from the worldwide isolates that have previously been identified and published. The nucleotide sequences of the MS2 and MS10 microsatellites analyzed in this study have been deposited in the GenBank database under accession numbers (Submission KJ700413 [MS10] and KJ700414 [MS2]).

This new isolate was designated *NC-Argentina LP1* (*Neospora caninum*-Argentina La Plata1), based on the country and laboratory data where the isolation was achieved.

## 4. Discussion

This paper describes the first successful attempt of isolation of *N. caninum* from a congenitally infected asymptomatic calf from Argentina and the difficulties associated with demonstration of live parasites. There is no standardized protocol for *N. caninum* isolation and the technique often ends in unsuccessful results (Dubey and Schares, 2006). Material for isolation is usually provided from aborted fetuses and is often autolytic and contaminated, reducing the likelihood of achieving viable *N. caninum* isolates. Isolation of *N. caninum* from fresh neural tissues of congenitally infected, live calves is easier since the viability of the parasite is not compromised by autolysis (Regidor-Cerrillo et al., 2008). Based on other isolation reports (Okeoma et al., 2004; Regidor-Cerrillo et al., 2008), we selected five asymptomatic calves with high precolostral serological titers for *N. caninum* from a dairy farm with endemic neosporosis to attempt isolation. Therefore, the material used was fresh, with minimal signs of autolysis. Additionally, *N. caninum* congenitally infected calves are expected to develop tissue cysts, which are more resistant to autolysis and trypsin/pepsin digestion compared to tachyzoites (Dubey and Schares, 2006; Rojo-Montejo et al., 2009). Developing a better method for parasite concentration may increase the chances of obtaining an isolate, especially in cases with low parasite load in asymptomatic calves. The protocol followed in this work used 0.5% trypsin digestion, and the resulted material was resuspended into a small amount of physiological solution. This concentration method used for mice inoculation may have increased the probability of isolating *N. caninum*. However, isolation was only successful in 1 of the 5 calves processed. Cellular immunity is critical for the control of intracellular parasite infections, and immunosuppressed inbred strains of mice are ideal for *Neospora*-isolation (Dubey and Schares, 2006). In this work, we used GKO mice for *in vivo* isolation which have a deficient cellular immune response, thus favoring the development of the disease. Mice inoculated with NC-Argentina LP1 isolate lived an average of 18–20 days. Many mammalian cell lines have been used for *in vitro* isolation, but there is no record for a specific preference of *N. caninum* toward a particular cell line (Lei et al., 2005). The NC-Argentina LP1 isolate grew well in *VERO* cells, and the early detection of tachyzoites might have been facilitated by the high number of tachyzoites obtained from the mouse peritoneal cavity used to infect cells.

Microsatellite sequences have been shown to be the most suitable polymorphic markers for the typing of *N. caninum* isolates, and have been applied to genetically characterize isolates obtained from cattle and canines worldwide (Brom et al., 2014). In the present work, MLST allowed us to characterize the NC-Argentina LP1 isolate, resulting in a unique distinctive microsatellite pattern genetically different from all other *N. caninum* isolates yet reported.

Most worldwide *N. caninum* isolates have been obtained from animals with clinical signs or from aborted fetuses. *Neospora caninum* isolation from infected but asymptomatic bovines is rare, since the animal has to be euthanized strictly for isolation purposes. Virulence differences between isolates from symptomatic and asymptomatic bovines have been reported. For instance, NC-Spain 1H, isolated from an infected but asymptomatic calf, exhibited low virulence in both mice and cattle models (Rojo-Montejo et al., 2009). Furthermore, inoculation with live NC-Spain 1H in heifers prior to breeding resulted in a reduced occurrence of *N. caninum*-associated abortion and vertical transmission (Rojo-Montejo et al., 2013). Similar findings were observed with NC-Nowra isolate, also obtained from an asymptomatic calf (Weber et al., 2013). Economic analyses have proposed that a vaccine is the most cost-effective approach to control the disease; however, up to date there is no effective commercial vaccine available. Consequently, there is an urge to study new candidates for vaccine development, and naturally attenuated *N. caninum* bovine isolates are promising for the design of live vaccines to control abortion and infection in cattle. This study describes the first successful isolation and genetic characterization of a *N. caninum* bovine isolate obtained in Argentina and may be a suitable candidate for vaccine design to control bovine neosporosis.

## Acknowledgements

The authors would like to thank Dr. E. Morrel, Mrs. T.L. Lischinsky, Mrs. D.B. Cano, and Mr. I. Ercoli for their excellent technical assistance and Dr. J.P. Dubey for helpful comments on the manuscript. Financial support for this study was provided by CONICET, through PIP 2012–2014 N° 112-20110100488.

## Conflict of interest

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of this article.

## References

Basso, W., Venturini, L., Venturini, M.C., Hill, D.E., Kwok, O.C., Shen, S.K., et al., 2001. First isolation of *Neospora caninum* from the feces of a naturally infected dog. J. Parasitol. 87, 612–618.

Basso, W., Schares, S., Bärwald, A., Herrmann, D.C., Conraths, F.J., Pantchev, N., et al., 2009. 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.

Basso, W., Moré, G., Quiroga, M.A., Balducchi, D., Schares, G., Venturini, M.C., 2014. *Neospora caninum* is a cause of perinatal mortality in axis deer (*Axis axis*). Vet. Parasitol. 199, 255–258.

Brom, P.R., Regidor-Cerrillo, J., Collantes-Fernández, E., Ortega-Mora, L.M., Guimarães, M.S., da Silva, A.C., 2014. Genetic characterisation of *Neospora caninum* strains from clinical samples of zebuine foetuses obtained in abattoirs in Goiás, Brazil. Vet. Parasitol. 204, 381–387.

Campero, C.M., Anderson, M.L., Conosciuto, G., Odriozola, H., Bretschenider, G., Poso, M.A., 1998. *Neospora caninum* associated abortion in dairy herd in Argentina. Vet. Rec. 143, 228–229.

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.

Dubey, J.P., Schares, G., 2006. Diagnosis of bovine neosporosis. Vet. Parasitol. 141, 1–34.

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

Lei, Y., Davey, M., Ellis, J.T., 2005. Attachment and invasion of *Toxoplasma gondii* and *Neospora caninum* to epithelial and fibroblast cell lines in vitro. Parasitology 131, 583–590.

Locatelli-Dittrich, R., Thomaz-Soccol, V., Richartz, R.R.T.B., Gasino- Joineau, M.E., vander Vinne, R., Pinckney, R.D., 2004. Isolamento de *Neospora caninum* de feto bovino de rebanho leiteiro no Parana. Rev. Bras. Parasitol. Vet. 13, 103–109.

Moore, D., Reichel, M., Späth, E., Campero, C., 2013. *Neospora cani*num causes severe economic losses in cattle in the humid pampa region of Argentina. Trop. Anim. Health Prod. 45, 1237–1241.

Moore, D.P., 2005. Neosporosis in South America. Vet. Parasitol. 127, 87–97.

Müller, N., Zimmermann, V., Hentrich, B., Gottstein, B., 1996. Diagnosis of *Neospora caninum* and *Toxoplasma gondii* infection by PCR and DNA hybridization immunoassay. J. Clin. Microbiol. 34, 2850–2852.

Okeoma, C.M., Williamson, N.B., Pomroy, W.E., Stowell, K.M., Gillespie, L.M., 2004. Isolation and molecular characterisation of *Neospora caninum* in cattle in New Zealand. N. Z. Vet. J. 52, 364–370.

Paré, J., Hietala, S.K., Thurmond, M.C., 1995. Interpretation of an indirect fluorescent antibody test for diagnosis of *Neospora* sp. infection in cattle. J. Vet. Diagn. Invest. 7, 273–275.

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.

Regidor-Cerrillo, J., Gómez-Bautista, M., Pereira-Bueno, J., Aduriz, G., Navarro-Lozano, V., Risco-Castillo, V., et al., 2008. Isolation and genetic characterization of *Neospora caninum* from asymptomatic calves in Spain. Parasitology 135, 1651–1659.

Rojo-Montejo, S., Collantes-Fernández, E., Regidor-Cerrillo, J., Alvarez-García, G., Marugan-Hernández, V., Pedraza-Díaz, S., et al., 2009. Isolation and characterization of a bovine isolate of *Neospora caninum* with low virulence. Vet. Parasitol. 159, 7–16.

Rojo-Montejo, S., Collantes-Fernández, E., Pérez-Zaballos, F., Rodríguez-Marcos, S., Blanco-Murcia, J., Rodríguez-Bertos, A., et al., 2013. Effect of vaccination of cattle with the low virulence NC-Spain 1H isolate of *Neospora caninum* against a heterologous challenge in early and mid-gestation. Vet. Res. 44, 106.

Venturini, M.C., Bacigalupe, D., Venturini, L., Rambeaud, M., Campero, C., Moore, D., et al., 2000. Detección de *Neospora caninum* en ratones inoculados con cerebros de fetos bovinos abortados. XXI Congreso Mundial de Buiatría, Uruguay.

Weber, F.H., Jackson, J.A., Sobecki, B., Choromanski, L., Olsen, M., Meinert, T., et al., 2013. On the efficacy and safety of vaccination with live tachyzoites of *Neospora caninum* for prevention of *Neospora*-associated fetal loss in cattle. Clin. Vaccine Immunol. 20, 99–105.