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RESEARCH NOTE

Isolation and biological characterisation of a new isolate of *Neospora caninum* from an asymptomatic calf in Brazil

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Abstract

Neospora caninum is a tissue-cyst forming parasite that has been recognized worldwide as a cause of abortion in cattle. Despite the ubiquitous distribution of this parasite and its broad range of hosts, the number of *N. caninum* isolates obtained to date is limited. In addition, the majority of isolates have been obtained from clinically affected hosts, therefore potentially biasing this population towards more virulent isolates. This report describes the isolation and biological characterisation of a new *N. caninum* isolate, Nc-Goiás 1, obtained from an asymptomatic, naturally infected calf from Brazil. This new isolate was identified as a member of the *N. caninum* species by polymerase chain reaction (PCR) using specific primers based on the *N. caninum* internal transcribed spacer 1 (ITS-1) sequence, and was genetically identified at multiple loci using microsatellite analysis. Finally, a pathogenicity study was conducted in a BALB/c mice model. All Nc-Goiás 1-infected mice survived without exhibiting any clinical signs. Further pathogenic characterisation of this isolate suggested that Nc-Goiás 1 is less virulent than other *N. caninum* isolates (Nc-Liv and Nc-1) studied in this mouse model. This is the first report of the isolation and biological characterisation of *N. caninum* from an infected but clinically healthy calf in South America.

Keywords

Neospora caninum, isolation, cattle, genetic characterisation, pathogenic characterisation, Brazil

Neospora caninum is a tissue-cyst forming coccidian parasite that is recognized as a cause of neuromuscular disease in dogs and neonatal mortality and abortion in cattle. Currently, *N. caninum* is considered a major cause of infectious bovine abortion worldwide, causing important economic losses to the cattle industry (Dubey *et al*. 2007). *N. caninum* infection may occur by horizontal transmission when cattle ingest sporulated oocyst shed by the definitive host (canids), although several reports have shown the importance of the endogenous congenital transmission from a persistently infected dam to foetus (Trees and Williams 2005). Infection in a pregnant animal may result in abortion, or in the birth of a weak calf, although in the majority of cases a clinically healthy but persistently infected calf is born. Different factors affect the outcome of neosporosis during the pregnancy, including the quantity and duration of parasitaemia in the recrudescence and the effectiveness of both the foetal and maternal immune response against the parasite (Innes *et al*. 2005, Collantes-Fernández *et al*. 2006b, Dubey *et al*. 2006). Additionally, other factors such as strain virulence may play an important role.

Biological characterisation of different laboratory-isolates of *N. caninum* has shown extensive genetic diversity (Schock *et al*. 2001; Regidor-Cerrillo *et al*. 2006, 2008) and significant variations in their *in vivo* pathogenicity and *in vitro* growth characteristics (Atkinson *et al*. 1999, Schock *et al*. 2001, Miller *et al*. 2002, Pérez-Zaballos *et al*. 2005, Collantes-Fernández *et al*. 2006a). Despite this, most of these laboratory-isolates have been obtained from either cattle or dogs with clinical signs, stillbirth calves, or bovine aborted foetuses (Dubey *et al*. 2007). Thus, new approaches are needed to determine the

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actual biological diversity of this parasite, at least of isolates that are obtained from naturally-infected, healthy cattle.

Recently, the isolation of a considerable number of *N. caninum* isolates from clinically healthy calves in Spain was reported (Regidor-Cerrillo *et al*. 2008, Rojo-Montejo *et al*. 2009). Here, we describe the isolation and biological characterisation of a new *N. caninum* isolate, Nc-Goiás 1, obtained from an asymptomatically, infected calf in Brazil.

The calf used for isolation was selected from a dairy herd in Nerópolis (Goiás, Brazil) that had a high intra-herd seroprevalence for *Neospora* infection (72%), as tested by an indirect fluorescent antibody test (IFAT) using 1:200 dilution as the cut-off (Álvarez-García *et al*. 2002). The calf was a 4-month-old Holstein-Friesian male with no clinical signs and a *N. caninum* antibody titre of 1:400. The dam was also seropositive for *N. caninum* at 4 months post-partum, with an IFAT titre of 1:200.

The calf was sacrificed and its brain was aseptically removed during the necropsy. No macroscopic abnormalities were noted. Brain sections of approximately 5 to 15 g were placed in PBS buffer containing 2% antibiotic-antimycotic solution (Gibco BRL, Paisley, UK) and maintained at 4°C until they were used for mice inoculation. *N. caninum* infection was confirmed in the calf brain sections by nested-PCR specific for the *N. caninum* internal transcribed spacer 1 (ITS-1) region (see below). Two tissue samples from each section were analyzed and parasite DNA was detected in 60% of the samples assayed, which originated from three of the different five brain sections collected. After tissue analysis, homogenized samples of ITS-1-PCR-positive brain portions were used to inoculate intraperitoneally three C57BL/6 IFN-γ KO mice. Mice with severe clinical signs (emaciation and lethargy) were sacrificed in a CO_2 chamber and their peritoneal cavities were flushed. Peritoneal washes were immediately inoculated onto a 24 hour cell monolayer culture of MARC-145 cells. Then, the mouse brains were aseptically removed and a portion was used for *in vivo* maintenance of the isolate until the cryopreservation of the parasite was carried out, as described (Regidor-Cerrillo *et al*. 2008). The isolation cultures were maintained with successive passages onto a fresh monolayer of cells every 4–7 days, as outlined by Regidor-Cerrillo *et al*. (2008).

Genomic DNA was extracted from 20 mg of either calf brain or murine brain tissues and also from 10^7 purified Nc-Goiás 1 tachyzoites using the Real Pure Extracción DNA Genómico kit (Durviz, Valencia, Spain), according to the manufacturer's instructions. Detection of parasite DNA was performed by a nested PCR specific for the ITS-1 region of *N. caninum* as described by Buxton *et al*. (1998), using the amplification conditions and analytic procedures previously described (Regidor-Cerrillo *et al*. 2008). In addition, tachyzoite DNA was tested by PCR and fragment analysis for 12 microsatellite markers to genetic characterisation, as previously described by Pedraza-Díaz *et al*. (2008). Only MS7 and MS10 markers were amplified and sequenced as was reported by Regidor-Cerrillo *et al*. (2006). DNA sequences were deposited in the GenBank database under accession number EU848549-EU848550.

Pathogenicity studies of the new isolate were performed using a BALB/c mouse model as described previously by Collantes-Fernández *et al*. (2006a). Briefly, groups of female 6-week-old mice (Harlan Interfauna Ibérica, Barcelona, Spain) were inoculated intraperitoneally with PBS (control) or with 10^6 Nc-Goiás 1 purified tachyzoites harvested from cell cultures. Mice were examined daily for clinical signs compatible with neosporosis. Five mice were randomly sacrificed with CO_2 gas on days 1, 2, 4, 8, 16 and 64 post-inoculation (p.i.) and samples were harvested from ten mice on day 32 p.i. Three mice from the control group were also sacrificed on these days. Blood samples were collected using EDTA tubes. Plasma and peripheral blood cells were obtained by centrifugation at 2000 *g* for 10 min. Plasma was aliquoted and preserved at –80°C for ELISA and peripheral blood cells were stored at 4°C for less than 24 h until genomic DNA extraction and PCR analysis. Brains and lungs were also collected under aseptic conditions and frozen at –80°C until DNA was extracted.

The temporal dissemination profile of the parasite was determined by ITS-1 PCR of DNA extracted from peripheral blood cells, lung, and brain samples. In addition, brain samples containing parasite DNA were tested by real-time PCR, as described by Collantes-Fernández *et al*. (2002). Parasite load was expressed as parasite number per microgram of host DNA. *N. caninum*-specific IgG2a and IgG1 isotypes were determined by ELISA using soluble *N. caninum* tachyzoite antigen as described by Collantes-Fernández *et al*. (2006a).

Differences in the PCR detectability of parasite DNA were analyzed, grouping data from days 1–8 p.i. (early phase) and from days 16 and 64 p.i. (chronic phase) and then comparing these two groups using the Fisher exact *F*-test. Variations in parasite loads in brain tissues were analyzed using the Kruskal-Wallis test, grouping data by day p.i. Finally, a Student *t*-test and 1-way ANOVA test, followed by Duncan's Multiple Range test, were employed to compare plasma anti-*N. caninum* antibody levels. A value of $P<0.05$ was considered statistically significant. Statistical analyses were performed using Statgraphics Plus version 5.1 software (Statpoint, Inc., Herndon, VA, USA).

At 22 to 42 days p.i., all mice inoculated with PCR-positive brain tissues from the infected calf showed clinical signs compatible with neosporosis such as emaciation, rough hair coats, wasting, ataxia, paraplegia, and lethargy. All brain samples from mice inoculated with calf tissues tested by ITS-1-PCR were positive, confirming *Neospora* infection. In addition, identical signs were observed in all IFN-γ KO mice used to maintain the parasite *in vivo* (7 mice), although the signs appeared at 6 to12 days p.i.

Few *Neospora*-like tachyzoites and parasite vacuoles were microscopically observed at 56 days p.i., after 9 blind passages in cell culture. The number of tachyzoites gradually increased in subsequent passages, and greater than 10^6 tachyzoites/ml were counted in a Neubauer chamber at 16 passages. DNA ex-

Table I. Assignment of alleles and allelic profile obtained for the Nc-Goiás 1 isolate

	Nc-Goiás 1										
Microsatellite loci	MS1A	MS1B	MS2	MS4	MS5	MS6A	MS6B	MS7	MS8	MS10	MS12	MS21
Allele no.	1	2	2	4	9	3	2	1	7	15	2	1
Repeat length	$(TA)_{60}$	$(AT)_{13}$	$(AT)_{22}$	$(AT)_{18}$	$(TA)_{20}$	$(TA)_{14}$	$(AT)_{12}$	$(TA)_{10}$	$(AT)_{12}$	$(ACT)_6$-$(AGA)_{16}$-$(TGA)_9$	$(GT)_{16}$	$(TACA)_{10}$

tracted from tachyzoites harvested in cell cultures was also positive by ITS-1-PCR. This isolate was called Nc-Goiás 1.

Microsatellite analysis of the Nc-Goiás 1 isolate showed that most of the alleles detected for each of 12 markers had been identified on different isolates in previous studies (Regidor-Cerrillo *et al*. 2006, 2008), although new alleles were described for microsatellites MS5 and MS10 (Table I). Thus, multilocus analysis showed a unique profile for the Nc-Goiás 1 isolate.

In the BALB/c bioassay, all mice inoculated with PBS or Nc-Goiás 1 tachyzoites survived until the end of the experiment and none showed any clinical signs compatible with neosporosis. *Neospora*-DNA was consistently detected in peripheral blood of Nc-Goiás 1-infected mice from days 1 to 4 p.i. and sporadically on days 32 and 64 p.i. (only detected in one mouse on each of these days). In the lungs, parasite DNA was amplified from days 1 to 8 p.i. and sporadically on day 16 p.i. In the brain, parasite DNA was amplified from day 8 p.i. until the end of the experiment (Table II).

Comparison of PCR-detection frequencies showed that parasite DNA was consistently detected in the blood and lungs during the early phase of infection, whereas detection of parasite DNA in the brain predominated at the chronic phase ($P<0.05$, Fisher *F*-test). The highest individual parasite numbers in the brain were detected during the chronic phase. However, no differences in the parasite loads were observed on day 8 to 64 p.i. ($P>0.05$, Kruskal-Wallis H test) (Fig. 1).

When the infected group of mice was compared with the PBS-inoculated group, IgG1 and IgG2a isotypes showed a significant increase on days 16 and 8 p.i., respectively ($P<0.05$, Student *t*-test). IgG1 levels peaked on day 32 p.i., and IgG2a levels increased gradually until day 32 p.i., presenting a plateau at day 64 p.i. ($P = 0$, 1-way ANOVA, followed by Duncan test) (Fig. 2A). Analysis of the IgG1/IgG2a ratio showed a predominance of IgG2a on days 8 and 16 p.i. (IgG1/IgG2a<1), whereas higher IgG1 levels were detected on day 32 followed by day 64 p.i. (IgG1/IgG2a>1) (Fig. 2B) ($P = 0$, 1-way ANOVA, followed by Duncan test).

Table II. Detection of *N. caninum* DNA by nested-PCR in the blood, lungs and brain from BALB/c mice inoculated with 10^6 Nc-Goiás 1 tachyzoites

Days p.i.	Blood	Lung	Brain
1	4/5[a] (80%)[b]	5/5 (100%)	0/5 (0%)
2	5/5 (100%)	4/5 (80%)	0/5 (0%)
4	3/5 (60%)	3/5 (60%)	0/5 (0%)
8	0/5 (0%)	4/5 (80%)	2/5 (40%)
16	0/5 (0%)	1/5 (20%)	3/5 (60%)
32	1/10 (10%)	0/10 (0%)	7/10 (70%)
64	1/5 (20%)	0/5 (0%)	2/5 (40%)

[a]Fractions represent the number of mice positive by nested PCR/number tested. [b]Percentage of detection.

In this study, an improved isolation method from asymptomatic calves was employed for the isolation of Nc-Goiás 1 using immunocompromised mice, such as IFN-γ KO mice, as step prior to parasite isolation in cell cultures (Regidor-Cerrillo *et al*. 2008). The clinically healthy but *Neospora*-infected calf was selected for isolation according to positive serological analysis and prior detection of the parasite by nested-PCR in brain tissues. In this case, endogenous transmission was likely the route of infection in the calf because IFAT analysis of the dam was also positive for *N. caninum*. Nevertheless, the horizontal transmission route could not be ruled out because exposure to parasites was evaluated four months after his birth, leaving a time window for horizontal transmission.

The new isolate obtained in cell culture was identified as *Neospora* sp. by ITS-1 nested-PCR. The microsatellite analysis of the Nc-Goiás 1 isolate displayed a unique multilocus profile, different from any *N. caninum* isolate profile described

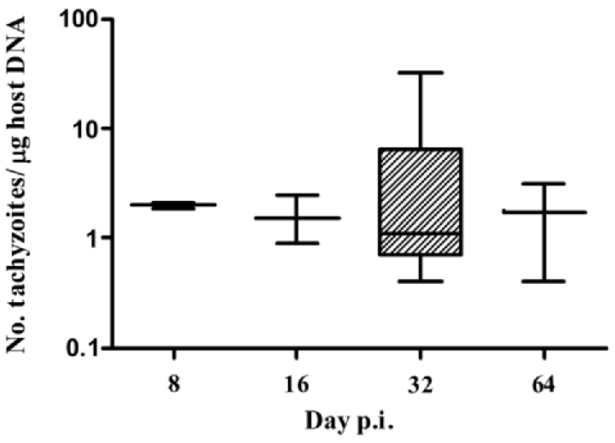

Fig. 1. Box-plot graph representing the maximum and minimum, lower and upper quartiles, and median values for brain parasite burden in BALB/c Nc-Goiás 1-infected mice from days 8 to 64 p.i.

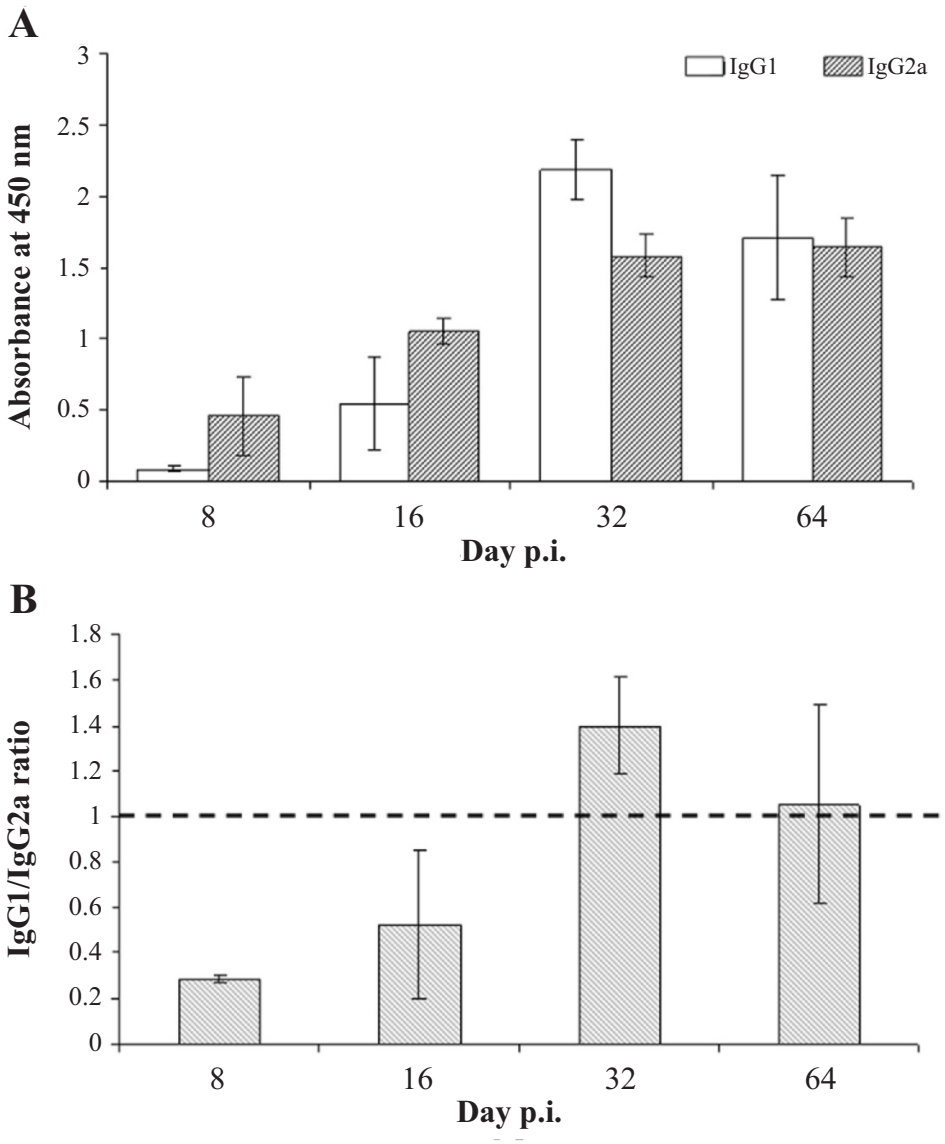

Fig. 2. Bars represent the mean absorbance of anti-*N. caninum* IgG1 and IgG2a isotypes (panel **A**) and ratio of IgG1/IgG2a (panel **B**) from BALB/c mice inoculated with 10^6 Nc-Goiás 1 tachyzoites from days 8 to 64 p.i. Error bars represent the SD. In panel **B**, the discontinuous line marks identical IgG1 and IgG2a levels (IgG1/IgG2a = 1). Data from days 1, 2 and 4 p.i. are not represented because anti-*N. caninum* isotype levels were not significantly different from those found in the PBS-infected mice group

in previous studies (Regidor-Cerrillo *et al*. 2006, 2008; Rojo-Montejo *et al*. 2009), confirming once more the high discrimination power of these genetic tools and the extensive genetic diversity of *N. caninum*.

Finally, a bioassay in BALB/c mice was employed to determine the pathogenicity of the Nc-Goiás 1 isolate (Collantes-Fernández *et al*. 2006a). In contrast to Nc-Liv and Nc-1 infections, no clinical signs were exhibited by mice inoculated with the Nc-Goiás 1. Even though the temporal tissue dissemination of the parasite observed in infected mice was very similar to that of the Nc-Liv and Nc-1 isolates. Nc-Goiás 1 distribution was characterised by an early phase, exhibiting parasitaemia and DNA detection in the lungs, and a chronic phase, characterised by parasite localization in the brain (Collantes-Fernández *et al*. 2006a). Importantly, the median parasite load by day in the brain of Nc-Goiás1-infected mice was clearly lower (≤ 2 tachyzoites/µg host tissue) than the load determined in Nc-1 and Nc-Liv-infected mice (Collantes-Fernández *et al*. 2006a). The kinetics of IgG1 and IgG2a production also showed a similar profile to the rates of antibody production in mice infected with Nc-Liv and Nc-1 isolates (Collantes-Fernández *et al*. 2006a), namely the predominance of IgG2a and IgG1 responses for the early and chronic phase of infection, respectively. These results indicate that Nc-Goiás 1 is apparently less virulent than some isolates obtained from clinically affected animals, such as Nc-SweB1, Nc-Liv and Nc-1 (Atkinson *et al*. 1999, Collantes-Fernández *et al*. 2006a). This was also described for other isolates obtained from

asymptomatic calves, such as JPA1, Nc-Nowra or Nc-Spain 1H (Shibahara *et al*. 1999, Miller *et al*. 2002, Rojo-Montejo *et al*. 2009). Further studies with a higher number of isolates obtained from infected but clinically healthy animals using natural-host infection models are needed to determine the real influence of virulence inherent to the isolate on the outcome of *N. caninum* infection in cattle and the differences between isolates from clinically affected and healthy animals.

This report describes the isolation and the biological characterisation of a new *N. caninum* isolate obtained from a Brazilian calf. In Brazil, *in vitro* isolations of *N. caninum* have been obtained from a dog (Gondim *et al*. 2001) and water buffaloes (Rodrigues *et al*. 2004). Two studies report parasite isolation from a blind calf (Locatelli-Dittrich *et al*. 2003) and from a 7-month-old aborted bovine foetus (Locatelli-Dittrich *et al*. 2004). To our knowledge, Nc-Goiás 1 is the first isolate obtained from an infected but clinically healthy calf in South America.

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