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Isolation and characterization of a bovine isolate of *Neospora caninum* with low virulence.

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Abstract

Neospora caninum tachyzoites were isolated from the brain of an asymptomatic naturally infected calf with precolostral-specific antibodies. The new isolate, named Nc-Spain1H, was identified as a member of the *N. caninum* species based on its internal transcribed spacer 1 (ITS-1) sequence and was genetically characterized using microsatellite markers. Multilocus analysis showed that Nc-Spain1H was genetically different from other *N. caninum* isolates. We compared the *in vitro* tachyzoite yield and viability rate of the Nc-Spain1H and Nc-1 isolates in a plaque assay. The lower tachyzoite yields displayed by Nc-Spain1H were complemented with a significantly lower viability rate. Moreover, in an *in vitro* tachyzoite-bradyzoite stage conversion assay, the percentage of Nc-Spain1H bradyzoite conversion was similar to that of the cystogenic isolate Nc-Liv, with the exception that Nc-Spain1H produced only intermediate bradyzoites. The pathogenicity of Nc-Spain1H was examined in BALB/c mice, and the results demonstrated that Nc-Spain1H failed to induce clinical signs or mortality and that no parasite DNA was detected in the brain during the chronic stage of infection. In a pregnant mouse model, Nc-1 infection resulted in high transplacental transmission, leading to a high neonatal mortality rate over time. In contrast, the offspring survival rate from Nc-Spain1H-infected dams was almost 100%, and *N. caninum* DNA was detected in only one pup. These data show that Nc-Spain 1H appears to be a low virulence isolate and may be a suitable candidate for live vaccine development.

Keywords: *Neospora caninum*, low virulent isolate, biological characterization.

1. Introduction

Neospora caninum is a cyst-forming coccidian parasite that is closely related to *Toxoplasma gondii* and has been recognized worldwide as a cause of neuromuscular disease in dogs and abortion in cattle (Dubey et al., 2006). The predominant route of transmission of the parasite in cattle is considered to be transplacental and may play a significant role in maintenance of the infection within a herd. Postnatal transmission, due to ingestion of sporulated *N. caninum* oocysts, can contribute to the sustainability of *N. caninum* infection, and it has been associated with abortion outbreaks (Wouda, 2007). Fetuses may die *in utero*, be resorbed, mummified, autolyzed, stillborn, born alive with clinical signs, or born clinically normal but with chronic infection (Dubey and Schares, 2006). Advances concerning the *N. caninum* life cycle have proven that dogs and coyotes are both intermediate and definitive hosts, whereas cattle and other mammals are natural intermediate hosts (Dubey et al., 2006). *N. caninum* has been isolated from dogs, cattle, sheep, water buffaloes, and white tailed deer (Dubey et al., 2006; Dubey et al., 2007).

Various authors have described the existence of the intra-specific variability of *N. caninum*, since differences in *in vitro* growth and virulence in mouse models have been observed among different isolates (Atkinson et al., 1999; Schock et al., 2001; Quinn et al., 2002; Miller et al., 2002; Pérez-Zaballos et al., 2005; Collantes-Fernández et al. 2006). In fact, the Nc-Nowra isolate, which was obtained from a congenitally infected calf, demonstrated low virulence in mice (Miller et al., 2002). Such intra-specific diversity may be associated with variation in the clinical presentation of disease. With regard to variability in the clinical presentation, little is known about differences among *N. caninum* isolates from symptomatic or asymptomatic animals, since a limited number of isolates

have been described and most characterized isolates were obtained from animals with clinical signs. In addition, analyses of the genetic diversity of *N. caninum* are limited. Random Amplified Polymorphic DNA-PCR (RAPD-PCR) and sequence analysis of rDNA internal transcribed spacer 1 (ITS-1) regions have been used to demonstrate intra-species diversity (Atkinson et al., 1999; Davison et al., 1999; Spencer et al., 2000; Schock et al., 2001; Gondim et al., 2004), but these techniques utilize genomic regions that are insufficiently polymorphic to differentiate between *N. caninum* isolates. The microsatellite technique has recently been used to demonstrate significant genetic diversity within *N. caninum*, enabling detailed studies of the genetic complexity of *N. caninum* infections (Regidor-Cerrillo et al., 2006).

This report describes the isolation and characterization of a new isolate from an asymptomatic, naturally infected calf that was designated Nc-Spain1H and showed low virulence in mice. Genetic characterization was carried out by ITS-1 sequencing and microsatellite marker analysis, and antigenic features were determined by western blot. The *in vitro* behavior of the Nc-Spain1H isolate was studied by evaluating tachyzoite yield and viability, as well as bradyzoite development in cell cultures. Finally, pathogenicity in a BALB/c mouse model was also investigated.

2. Materials and Methods

2.1. Nc-Spain 1H isolation procedure

2.1.1. Case report

The parasite was isolated from a clinically healthy Holstein-Friesian 2-week-old female calf with a precolostral *N. caninum* antibody titer of 1:500, as estimated by an indirect fluorescent antibody test (Álvarez-García et al., 2002). The dam of the calf was *N.*

caninum-positive and came from a dairy herd in Madrid (Spain) with high intra-herd *N. caninum* seroprevalence. The calf was sacrificed, and the brain was analyzed to confirm *N. caninum* infection. A specific ITS-1 nested-PCR test (Buxton et al., 1998) amplified *N. caninum* DNA, and histological examination revealed lesions compatible with protozoan infection in the brain (Barr et al., 1991), characterized by isolated gliosis foci and perivascular cuffing in all bovine sections analyzed.

2.1.2. Bioassay in nude mice and cell culture

PCR-positive calf brain portions were homogenized, filtered in sterile gauze, and centrifuged at 1350 x *g* for 15 min. The sediment was resuspended in PBS with antibiotics (2000 U.I/ml penicillin G and 200 µg/ml of streptomycin) (Gibco) and four 4-week-old female nude mice (Charles River, Barcelona, Spain) were inoculated intraperitoneally (i.p.) with homogenized calf brain tissue equivalent to 5 g. The mice were examined daily, and all animals showed clinical neosporosis at 32 - 40 days post-inoculation (p.i.). Apathy was the first sign of disease, followed by wasting and inactivity. In addition, a nested-PCR test was performed on murine brain samples to confirm the presence of *N. caninum* DNA, and all samples tested were positive.

For *in vitro* cultivation, the mouse peritoneal cavity was flushed, and peritoneal washes were inoculated onto a 24-h cell monolayer culture of MARC-145 cells. The cell cultures were subsequently passaged onto a fresh monolayer cell culture every 4-7 days. *Neospora*-like tachyzoites were observed 24 days after the fourth passage in cell culture. This new isolate was called Nc-Spain1H. A sample of the Nc-Spain1H isolate was deposited in the *Culture Collection of Algae and Protozoa* (Oban, Scotland, UK) under the CCAP Accession Number 2051/2.

115 *2.2. Genetic characterization of the Nc-Spain1H isolate*

116 *2.2.1. ITS-1 sequencing*

117 NN1 and NN2 primers designed by Buxton et al. (1998) were used to amplify and
118 sequence the ITS-1 region from Nc-Spain1H, Nc-Liv (Barber et al., 1995), and Nc-SweB1
119 (Stenlund et al., 1997) isolates. Parasites were purified from the cell monolayer by rupture
120 with a 25-gauge needle, followed by centrifugation (1350 × *g*, 4 °C, 15 min). The pellet
121 was resuspended in cold PBS, washed once by centrifugation, and resuspended in 5 ml of
122 PBS, and parasites were separated from host cells on PD-10 columns (Amersham
123 Biosciences), as described previously (Hemphill, 1996). DNA was extracted from 10^8
124 tachyzoites as described below. Amplified products were visualized under UV light in a
125 1.5% agarose/ethidium bromide gel, purified using the Geneclean Turbo kit (Q-BIOgene,
126 Carlsbad, USA) according the manufacturer's instructions, and then directly sequenced
127 using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster
128 City, USA) and a 3730 DNA analyzer (Applied Biosystems) at the Unidad Genómica del
129 Parque Científico de Madrid. PCR products were sequenced twice in both directions.
130 Sequences were analyzed using the BioEdit Sequence Alignment Editor v.7.0.1
131 (Copyright© 1997-2004 Tom Hall, Ibis Therapeutics, Carlsbad CA 92008, USA).

132 *2.2.2. Microsatellite analysis*

133 Fifty nanograms of parasite DNA were used to amplify 13 microsatellites, as
134 previously described (Regidor-Cerrillo et al., 2006). PCR products were visualized under
135 UV light in a 2.5% NuSieve 3:1-agarose/ethidium bromide gel (Nu-Sieve 3:1, Cambrex
136 BioScience, USA), purified using the Exo-SAP IT kit (USB, USA) according the
137 manufacturer's instructions, and then directly sequenced and analyzed as described above.

138 *2.3. Western blot analysis*

139 Western blot analysis was performed under reducing conditions according to a
140 previously described procedure (Álvarez-García et al., 2002; Chávez-Velásquez et al.,
141 2005) with 2×10^7 tachyzoites/gel of purified Nc-Spain1H and Nc-1 isolates (Dubey et al.,
142 1988). Murine and bovine sera were tested at dilutions of 1:50 and 1:20, respectively. As
143 secondary antibodies, a rabbit polyclonal anti-mouse IgG (whole molecule) (1:500) (Sigma
144 Chemical Co.) and a mouse monoclonal anti-bovine IgG1 and IgG2 antibody (1:400)
145 (Hipra Laboratories S.A.; Gerona, Spain) were used. Murine plasma pools were made by
146 mixing an equal volume of plasma per mouse obtained on day 32 p.i. from BALB/c mice
147 infected with 10^6 and 10^7 Nc-Spain1H tachyzoites and mice infected with 10^6 Nc-1
148 tachyzoites from previous studies (Collantes-Fernández et al., 2006). A pool of plasma
149 from mice injected with PBS was also included as a negative control. Cattle serum samples
150 included precolostral serum from the naturally infected calf from which Nc-Spain1H was
151 isolated, precolostral positive sera from several field cases of congenitally infected calves,
152 and a precolostral negative serum sample as a control**.**

153 *2.4. "In vitro" behavior of the Nc-Spain1H isolate*

154 *2.4.1. Tachyzoite yield and viability assays*

155 For both the tachyzoite yield and viability assays, the reference Nc-1 isolate
156 obtained from a dog with clinical signs (Dubey et al., 1988) was employed as a control. For
157 this, Nc-1 tachyzoites were propagated under new culture conditions using MARC-145
158 cells. This shift from Vero cells to a new cell line was expected to homogenize cell passage
159 in Nc-1 (Pérez-Zaballos et al., 2005). Prior to the experiment, Nc-1 and Nc-Spain1H
160 tachyzoites were maintained *in vitro* by continuous passage in MARC-145 cell monolayers

as previously described (Pérez-Zaballos et al., 2005) to ensure healthy actively replicating parasites. The experiment was carried out using similar parasite passage numbers in MARC-145 cells for Nc-1 (passage no. 22) and Nc-Spain1H (passage no. 14). First, the tachyzoite yield was determined in MARC-145 monolayers grown in 25 cm^2 tissue culture flasks inoculated with Nc-1 and Nc-Spain1H isolates. The number of tachyzoites and, consequently, the infection dose were determined by Trypan blue exclusion followed by counting in a Neubauer chamber. An optimized multiplicity of infection (MOI) was used for each isolate, with the aim of estimating the tachyzoite yield of both isolates in optimal conditions. Thus, the differences observed between parasite strains are not simply a consequence of the infection efficiency. As a result, we obtained a maximum number of infected cells and parasite vacuoles 3.5 days post-infection, and then the number of both total and viable tachyzoites recovered was estimated for each isolate by Trypan blue exclusion. The experiment was performed twice, with individual samples assessed in triplicate. Second, the viability assay was carried out as a plaque assay. Thus, for each isolate, 100 tachyzoites were inoculated into a 24-well plate containing MARC-145 monolayers (six wells per isolate) grown on 10 mm coverslips. At day 5 p.i., the number of plaque forming tachyzoites per well were determined by counting the lysed areas utilizing a direct immunofluorescent test with rabbit antiserum developed against *N. caninum* (Nc-1 isolate), as described previously (Risco-Castillo et al., 2004).

2.4.2. Tachyzoite-bradyzoite stage conversion

This study determined the tachyzoite-bradyzoite conversion rate of Nc-Spain1H compared with the cystogenic Nc-Liv isolate. We compared Nc-Spain1H only with Nc-Liv because the Nc-1 tachyzoite-bradyzoite conversion rate is very low in cell culture (data not

184 shown). The tachyzoite-bradyzoite conversion rate was observed by culturing infected
185 MARC-145 cells with medium containing 70 μM SNP for up to 6.5 days. Control wells
186 were maintained with medium at pH 7.2 for 3 - 4 days (Risco-Castillo et al., 2004). Cell
187 cultures were inspected, and the medium was changed daily. Nc-Liv tachyzoites were
188 maintained at a 0.5:1 host-parasite ratio, whereas the Nc-Spain1H isolate was assayed at an
189 MOI of 2.5. At 3, 5, and 6.5 days after stress, the tachyzoite-bradyzoite conversion rate was
190 assessed using a double immunofluorescence assay. Identification of tachyzoite and
191 bradyzoite development can be discerned by labeling with antibodies directed against the
192 immunodominant *N. caninum* tachyzoite surface antigen NcSAG1 (Fuchs et al., 1998) and
193 antiserum against *T. gondii* bradyzoite antigen 1 (BAG1), which exhibits cross-reactivity
194 with *N. caninum* bradyzoites (McAllister et al., 1996). This technique has been broadly
195 utilized in previous *N. caninum* tachyzoite-bradyzoite conversion studies (Weis et al., 1999;
196 Vonlaufen et al., 2002; Risco-Castillo et al., 2004). Thus, coverslips were labeled with a
197 monoclonal mouse antibody directed against the tachyzoite surface antigen NcSAG1
198 (αSAG1) (1:2000) and a polyclonal rabbit antiserum raised against the intracytoplasmic
199 bradyzoite antigen BAG1 (αBAG1) (1:100) (Risco-Castillo et al., 2004) by incubation at
200 room temperature (RT) for 1 h. The incubation was followed by two brief washes and three
201 5 min washes in PBS containing 0.1% BSA, followed by incubation with 0.1 mg/ml 49,6-
202 diamidin-2-phenylindole (Sigma Chemical Co., St. Louis, Mo USA) and the appropriate
203 conjugates (Molecular Probes) (RT, 1h). The coverslips were washed as previously
204 described, including a final wash in distilled water. Finally, the coverslips were overlaid
205 with 40% glycerol and 2.5% 1,4-diazabicyclo[2,2,2]octane (Sigma Chemical Co.) in PBS.
206 Antibody binding was observed with a 100X oil-immersion objective on a fluorescence-

inverted microscope (Nikon Eclipse TE200). The experiment was performed twice, and individual samples were evaluated in triplicate. The tachyzoite-bradyzoite conversion rate was calculated by random counting of 10 fields per coverslip and comparing the percentage of BAG1-positive parasitophorous vacuoles (PV) containing either pure or intermediate bradyzoites versus total PV.

2.5. *Pathogenicity studies of the Nc-Spain 1H isolate in BALB/c mice*

2.5.1. Experiment 1.

Transmission of the parasite during pregnancy was examined in a pregnant BALB/c mouse model (López-Pérez et al. 2006; López-Pérez et al., 2008), and differences between the Nc-Spain 1H and Nc-1 isolates were studied. Eight-week-old female mice (Harlan Interfauna Ibérica, Barcelona, Spain) were mated for four nights following synchronization of ovulation using the Whitten effect (Whitten, 1957). The mice were inoculated subcutaneously with 2×10^6 tachyzoites of Nc-Spain1H, Nc-1, or PBS at mid-gestation (6-10 days of pregnancy). Gestation was evaluated by determining the weight of the mice on day 18 after the first night mated. Pregnant mice (≥25 g) were housed individually and allowed to carry their pregnancy to term. Neonates and dams were sacrificed with CO_2 gas on day 30 post-partum (P.P.). Litter size was defined as the number of pups delivered per dam. Body weight was determined every two days from 14 to 20 days P.P. to avoid excessive handling of the pups and their consequent rejection by the dams. Stillbirth was evaluated as the number of full-term dead pups at the time of birth. Neonatal mortality was considered as the number of dead pups from birth to 30 days P.P. Clinically affected mice were humanely sacrificed by CO2 inhalation. The vertical transmission rate was determined by evaluating the presence of the parasite in the lung or brain of neonates by nested-PCR.

Brain and lung samples were collected from sacrificed neonates and frozen at –80 ºC for DNA extraction. Blood samples from dams were collected by cardiac puncture, and the recovered sera were aliquoted and cryopreserved at -80 ºC for ELISA. Brains from dams were also removed and frozen (-80 ºC) until used for PCR amplification. The remaining mice that did not result in pregnancy were used to evaluate chronic *N. caninum* infection (Collantes-Fernández et al., 2006). They were sacrificed with CO_2 gas 30 days p.i., and sera and brains were recovered for ELISA and PCR testing, respectively, as described above.

2.5.2. Experiment 2.

An inoculum dose titration in the BALB/c mouse model (Collantes-Fernández et al., 2006) was performed in order to comprehensively investigate Nc-Spain1H pathogenicity. Six-week-old female mice (Harlan Interfauna Ibérica, Barcelona, Spain) were inoculated with 10^5, 10^6, or 10^7 Nc-Spain1H tachyzoites by i.p. injection of a final volume of 200 µl/mouse, as previously described (Collantes-Fernández et al., 2006). A control group of BALB/c mice was i.p. inoculated with 200 µl of PBS. Three random animals from each group were sacrificed with CO_2 gas on days 1, 2, 4, 8, 16, and 32 p.i., excluding the group inoculated with 10^7 tachyzoites, for which four mice were killed on days 1, 2, and 4. The day of infection was referred to as day 0, and animals were monitored daily. Blood samples (300-500 µl) were collected in EDTA tubes by cardiac puncture and centrifuged (2000 × *g*, 10 min), and the plasma was recovered, aliquoted, and cryopreserved at -80 ºC for ELISA and western blot analysis. Peripheral blood cells were stored at 4 ºC for no longer than 24 h for DNA extraction. Target organs (lungs and brains) were recovered under stringent aseptic conditions to avoid cross-contamination and then frozen (-80 ºC) until analyzed by *N. caninum* PCR amplification.

253 *2.6. DNA extraction and ITS-1 nested-PCR*

254 The GenomicPrep cell and tissue DNA isolation kit (Amersham Biosciences,
255 Uppsala, Sweden) was used to extract DNA from *N. caninum* tachyzoites and 10-20 mg of
256 each host tissue, and the GenomicPrep blood DNA isolation kit (Amersham Biosciences,
257 Uppsala, Sweden) was used to extract DNA from blood samples according to the
258 manufacturer's instructions.

259 Nested-PCR of the ITS-1 region of *N. caninum* was carried out with four oligonucleotides,
260 as described by Buxton et al. (1998). The secondary amplification product was visualized
261 by 1.8% agarose gel electrophoresis and ethidium bromide staining. To avoid false positive
262 reactions, DNA extraction, PCR sample preparation, and electrophoresis were performed in
263 separate rooms employing different sets of instruments, aerosol barrier tips, and disposable
264 gloves. Moreover, negative control samples were included in each set of DNA extractions
265 and PCR reactions.

266 *2.7. Humoral immune responses to experimental murine infections*

267 *N. caninum*-specific serum isotypes, IgG2a and IgG1, were determined by ELISA using
268 a soluble *N. caninum* tachyzoite antigen. Briefly, 96-well plates were coated with soluble
269 *N. caninum* tachyzoite antigen (0.5 µg in 100 µl/well), and diluted murine serum samples
270 (1:100) and anti-mouse IgG1 or IgG2a antibody (1:5,000; Southern Biotechnology,
271 Birmingham, AL, USA) were used as described previously (Collantes-Fernandez et al.,
272 2006). The serum isotype balance was evaluated via the IgG1/IgG2a ratio.

273 *2.8. Data analysis*

274 In *in vitro* assays, differences in tachyzoite yield and viability between isolates were
275 evaluated using the non-parametric Mann-Whitney *U*-test, and the percentages of PV

276 expressing BAG1 over time were compared by the Student's *t*-test. In the pathogenicity
277 studies, a one-way ANOVA followed by Duncan's Multiple Range test was employed to
278 compare serum anti-*N. caninum* antibody values, litter size, and neonate body weight.
279 Differences in PCR detectability and vertical transmission were analyzed by the Chi-square
280 and Fisher *F*-tests. Neonatal mortality was analyzed by the Kaplan-Meier survival method
281 (Bland and Altman, 1998) for estimation of the portion of surviving individuals 30 days
282 P.P. The log-rank statistical test was applied to compare the survival curves (Bland and
283 Altman, 2004). Statistical analysis was carried out using SAS 8.02 (SAS Institute, Cary,
284 NC, USA) and GraphPad Prism 4 v.4.03 (San Diego, CA, USA).

285 **3. Results**

286 *3.1. Genetic and immunogenic characterization*

287 No differences were detected among the ITS-1 sequences obtained from the Nc-
288 Spain1H, Nc-Liv, and Nc-SweB1 isolates in this study when they were compared with
289 those previously deposited in the GenBank database corresponding to Nc-Liv (accession
290 number AY259038) and Nc-SweB1 (accession number AY259038), with the possible
291 exception of one ambiguous base (K) discovered in the Nc-Spain1H and Nc-Liv sequences
292 at position 373. The nucleotide sequences of the Nc-Spain1H, Nc-Liv, and Nc-SweB1
293 isolates obtained in this study were deposited in the GenBank database under accession
294 numbers EU564165, EU564166, and EU564167**,** respectively.

295 In addition, alleles for each of 13 microsatellites used in a previous study (Regidor-
296 Cerrillo et al., 2006) were amplified and sequenced from the Nc-Spain1H isolate. Most
297 alleles detected in this isolate were previously identified in an analysis of nine isolates by
298 Regidor-Cerrillo et al. (2006), except for microsatellite markers MS3 and MS6A, for which

299 new alleles with 11 and 18 dinucleotide repeat units, respectively, were described (Table 1).
300 Thus, multilocus analysis showed a unique profile for the Nc-Spain1H isolate. The
301 nucleotide sequences of the microsatellites analyzed in this study have been deposited in
302 the GenBank database under the accession numbers specified in Table 1.

303 The antigenic profiles detected by western blotting were the same for plasma
304 samples collected from Nc-Spain1H- and Nc-1-inoculated mice. Precolostral positive calf
305 sera also recognized similar profiles in both cases. The pattern of antigen recognition
306 corresponded to the *N. caninum* immunodominant antigens previously described (Álvarez-
307 García et al., 2002), with molecular weights of 17-18, 34-35, 37, and 60-62 kDa (data not
308 shown). No antigens were detected when negative sera were analyzed.

309 *3.2. "In vitro" behavior of the Nc-Spain1H isolate*

310 The Nc-Spain1H isolate, when inoculated onto a cell monolayer, showed
311 cytopathogenic effects similar to the Nc-1 isolate, which is associated with the rupture of
312 infected cells. However, the Nc-1 isolate destroyed 80% of the cell monolayer, and many
313 tachyzoites were observed in lysed areas, whereas only 20% of the Nc-Spain1H-infected
314 monolayers were disrupted 3.5 days post-infection, subsequently revealing significantly
315 different tachyzoite yields ($P<0.01$, Mann Whitney *U*-test) (Fig. 1A). Nevertheless, clusters
316 of Nc-Spain1H tachyzoites were observed outside the cells. The lower parasite tachyzoite
317 yield displayed by Nc-Spain1H was complemented by a significantly lower viability rate
318 when compared with the Nc-1 isolate in a plaque assay ($P<0.01$, Mann Whitney *U*-test).
319 Five days post-infection, 17.3 % and 36.5 % of the plaque-forming tachyzoites were
320 observed in wells inoculated with the Nc-Spain1H and Nc-1 isolate, respectively.

The tachyzoite-bradyzoite conversion rate was estimated from 3 to 6.5 days after stress for both the Nc-Spain1H and Nc-Liv isolates. Both isolates showed a high rate of bradyzoite conversion, with similar percentages (P>0.05, Student *t*-test) (Fig. 1B). However, 6.5 days after stress, the Nc-Spain1H isolate produced only intermediate bradyzoites (SAG1- and BAG1-positive), whereas 3.4% of the PV in the Nc-Liv isolate contained pure bradyzoites (BAG1-positive). Once again, we observed that the monolayer infected with Nc-Liv was essentially destroyed 6.5 days after stress, but the monolayer infected with Nc-Spain1H was hardly damaged.

3.3. Pathogenicity studies of the Nc-Spain1H isolate in BALB/c mice

In experiment 1, 23.1% (6/26), 44.4% (12/27), and 37% (10/27) of the females inoculated with Nc-Spain1H, Nc-1, and PBS, respectively, became pregnant. No differences in litter size were observed among the groups (P>0.05, 1-way ANOVA) (Table 2), but there was an increase in the number of stillborn animals in infected groups compared to uninfected mice, being significant only for the Nc-1 infected group (P <0.05, χ^2). In addition, we found a higher number of PCR-positive stillborns in the Nc-1-infected group (72.7%, 8/11) compared to Nc-Spain1H-infected (0%, 0/5) and uninfected mice (0%, 0/2). The offspring in the Nc-1-infected group showed significantly lower body weight compared to Nc-Spain1H-infected and control mice from 14 to 20 days PP (P<0.05, one-way ANOVA and Duncan's post-test) (Fig. 2). Pups born to Nc-1-infected mice showed clinical signs (delayed hair coat development, rough hair coat, and neurological signs) and a high neonatal mortality rate (76.8%, 43/56), in contrast to the Nc-Spain1H-infected group, in which only one pup without clinical signs died during the observation period (0.5%, 1/20). No neonatal mortality was detected in control animals. The survival

344 percentages of the Nc-Spain1H infected group and PBS-inoculated mice were significantly
345 greater than that of the Nc-1 infected group (P<0.001, Log-rank test) (Fig. 3).
346 Subsequently, Nc-1 infection produced the highest vertical transmission rate (92.8 % of
347 pups), and 100 % of the dams transmitted the infection to at least one of the pups of their
348 litter compared to the Nc-Spain1H infected group, in which *N. caninum* DNA was detected
349 in the brain of only one pup (P<0.0001, χ^2) (Table 2). Among the dams, parasite DNA was
350 detected in 75% (9/12) of the brain samples from Nc-1-infected mice and 33.3% (2/6) of
351 those from Nc-Spain1H-infected mice. With regard to the presence of parasites in the
352 brains of mice that did not result in pregnancy, we detected *N. caninum* DNA in all samples
353 from the Nc-1-infected group (100%, 15/15) and in only one sample from the Nc-Spain1H-
354 infected group (5%, 1/20). Regarding IgG2a and IgG1 responses, specific antibody
355 responses were significantly higher in both infected groups than those in the non-infected
356 group (P<0.001, 1-way ANOVA, Duncan's post-test). Among mice that became pregnant
357 (Fig. 4A), the Nc-1-infected group showed significantly higher IgG1 and IgG2a levels than
358 the Nc-Spain1H-infected group (P<0.05, 1-way ANOVA, Duncan's post-test). Similarly, in
359 mice that did not result in pregnancy (Fig. 4B), the Nc-1-infected group demonstrated
360 higher IgG1 and IgG2a levels than Nc-Spain1H-infected group, but only IgG2a levels
361 showed significant differences (P<0.05, 1-way ANOVA, Duncan's post-test). In both
362 mouse models, all infected groups had a higher concentration of IgG1 than IgG2a
363 (IgG1/IgG2a ratio>1), but no significant differences were observed between groups (data
364 not shown).

365 In experiment 2, none of the infected mice showed clinical neosporosis or
366 succumbed during the follow-up period. *N. caninum* DNA was observed in peripheral

blood from 1 to 4 days p.i., in the lungs from 1 to 16 days p.i., and in the brain from 1 to 8 days p.i. (Table 3). The infection was dose-dependent, and higher doses resulted in increased parasite detection. Parasite DNA was not detected in target organs obtained from the control group. Regarding IgG2a and IgG1 responses (Fig. 5), mice inoculated with 10^7 tachyzoites demonstrated higher levels of IgG1 and IgG2a than the other groups on days 8, 16, and 32 p.i. ($P<0.0001$, 1-way ANOVA, Duncan's post-test). Mice inoculated with 10^5 tachyzoites had a higher concentration of IgG2a than IgG1 at all levels of infection (IgG1/IgG2a ratio<1), whereas, in the group inoculated with 10^6 tachyzoites, IgG2a predominated only at 8 days p.i. Groups inoculated with 10^7 tachyzoites showed a significant predominance in IgG1 levels (IgG1/IgG2a ratio>1) from 8 to 32 days p.i. (Fig. 5).

4. Discussion

Since the first isolation of *N. caninum* (Dubey et al., 1988), a limited number of isolates have been described and characterized. Moreover, most *N. caninum* studies have assessed isolates obtained from clinical cases. This could hamper our understanding of *N. caninum* pathogenesis, and isolate populations. *N. caninum* isolates of bovine origin have been obtained from aborted fetuses, stillborns, and apparently healthy animals (neonatal calves with precolostral *Neospora*-specific antibodies and two adult asymptomatic cows) (Dubey et al., 2007). It appears that the procedure utilized to isolate *N. caninum* from neural tissues of congenitally infected full-term calves is easier because tissue cysts, which are likely to be present, are relatively more resistant to autolysis than tachyzoites (Dubey and Schares, 2006). Based on this, we tried to obtain an isolate from a healthy congenitally infected calf with precolostral *Neospora*-specific antibodies. Obtaining and characterizing

390 isolates from animals with different clinical forms of disease is necessary to evaluate
391 correlations among parasite strains, genotypes, clinical manifestations, and pathogenicity.
392 Furthermore, the identification of *N. caninum* avirulent isolates could lead to the
393 development of live vaccines.

394 Identification and characterization of the new isolate was initially based on
395 amplification and sequencing of the *N. caninum* ITS-1 region using a specific PCR test.
396 ITS-1 sequence analysis has been used as an important tool for species characterization and
397 differentiation between *Neospora* spp. and closely related parasites. The ITS-1 sequences
398 obtained in this study identified the Nc-Spain1H isolate as a member of the *N. caninum*
399 species. Inter- and intra-strain variations in ITS-1 sequences between *N. caninum* isolates
400 have been described (Gondim et al., 2004); however, they are not appropriate for intra-
401 species differentiation. In contrast, microsatellite marker analysis proved to be a suitable
402 tool for the genetic analysis and discrimination of *N. caninum* isolates (Regidor-Cerrillo et
403 al., 2006). Thus, in the present study, 13 microsatellite markers were amplified and
404 sequenced, and multilocus analysis showed that Nc-Spain1H was genetically different from
405 the other *N. caninum* isolates tested. In fact, two new alleles for microsatellites MS3 and
406 MS6A were found. Analyses of the genetic diversity of *N. caninum* are limited. Further
407 comparisons of isolates from different origins could be of considerable interest in
408 determining how particular *N. caninum* genotypes might differ in their virulence and to
409 facilitate predictions of infection outcome. In addition, the microsatellite technique could
410 be useful in molecular epidemiological studies.

411 The antigenic specificity of the Nc-Spain1H isolate was also confirmed by western
412 blot using precolostral calf serum and plasma samples from mice inoculated with Nc-

Spain1H. When bovine sera and murine plasma samples were analyzed, immunodominant antigens of 17-18, 34-35, 37, and 60-62 kDa, which were previously identified (Alvarez-García et al., 2002), were recognized, and western blot revealed no major differences between the new Nc-Spain1H isolate and the reference Nc-1 isolate. Similar profiles were described by Atkinson et al. (1999), who analyzed extracts from Nc-Liv and Nc-SweB1 isolates by western blotting with sera from experimentally infected mice.

With the aim of investigating the *in vitro* behavior of the Nc-Spain1H isolate, we compared the tachyzoite yield of Nc-Spain1H and Nc-1 isolates, since the *in vitro* growth rate and invasion efficiency are common virulence characteristics in protozoan pathogens, and growth rate differences between isolates have been previously described in both *N. caninum* (Schock et al., 2001; Pérez-Zaballos et al., 2005) and *Toxoplasma gondii* (Saeij et al., 2005). A significantly higher tachyzoite yield was found for the Nc-1 isolate, and the number of plaque-forming tachyzoites counted in Nc-Spain1H cultures was significantly lower compared to that of the Nc-1 isolate in the viability assay. Additionally, in both *in vitro* assays, we observed that Nc-1 destroys the monolayer faster than Nc-Spain1H. Similar observations have been described for the three major strains of *T. gondii*, and this finding has been explained as due to a higher reinvasion rate of type I parasites. Extracellular type I parasites, regarded as "virulent", remain infectious for a longer period of time compared with type II or III strains (referred to as "avirulent"), and, consequently, they might be able to disseminate more efficiently to new cells after a previously infected cell is lysed (Saeij et al., 2005).

During *N. caninum* infection, parasite persistence is established in the CNS, where the parasite transforms into the tachyzoite-bradyzoite stage, which is walled off inside

436 tissue cysts (Buxton et al., 2002). In order to determine the bradyzoite conversion rate of
437 Nc-Spain1H, an *in vitro* tachyzoite-bradyzoite stage conversion assay was performed. The
438 stage conversion of Nc-Spain1H observed in cell culture was similar to that seen in a
439 cystogenic isolate of Nc-Liv, with the exception that Nc-Spain1H produced only
440 intermediate bradyzoites. This could potentially hamper parasite persistence since the
441 expression of specific pure bradyzoite antigens is a mechanism employed to evade the
442 immune response induced against fast replicating tachyzoites during acute infection. Our *in*
443 *vitro* observations correlate well with the results obtained in the *in vivo* assays. On one
444 hand, the low virulence of Nc-Spain1H observed in mice could be explained by decreased
445 parasite invasion of or a lower growth rate in host cells, which would lead to less efficient
446 parasite dissemination.

447 Mouse inoculation was employed to determine the variation in virulence among *N.*
448 *caninum* isolates. In previous studies, Nc-Liv was more virulent than Nc-1 and Nc-SweB1
449 (Atkinson et al., 1999; Quinn et al., 2002; Collantes-Fernández et al., 2006), and Nc-Nowra
450 and JPA1 isolates obtained from healthy, but congenitally infected calves, were associated
451 with low virulence in mice (Shibahara et al., 1999; Miller et al., 2002). We evaluated
452 parasite virulence in a pregnant mouse model (López-Pérez et al. 2006; López-Pérez et al.,
453 2008). An increased number of stillborn pups were observed in infected mice, but parasite
454 DNA was found only in stillborns from the Nc-1-infected group, suggesting that they may
455 have died as a consequence of infection. We were unable to detect parasite DNA in the
456 stillborn samples obtained from the Nc-Spain1H group. Moreover, infection with Nc-1
457 during pregnancy caused high transplacental transmission, leading to a high neonatal
458 mortality rate over time. In contrast, offspring from Nc-Spain1H-infected dams remained

clinically normal, the survival rate was almost 100%, and *N. caninum* DNA was detected in only one pup. In addition, parasite DNA was more often detected in brain samples from Nc-1-infected dams than Nc-Spain1H-infected dams. Among those mice that did not become pregnant, parasite DNA was detected in all animals infected with the Nc-1 isolate and only one of those infected with the Nc-Spain1H isolate. In another study, the Australian isolate Nc-Nowra was described as a low virulence isolate, although, when an inoculum of 10^6 tachyzoites per mouse was used, mortality was observed in 3 out of 10 infected mice and parasite DNA was detected in brain samples. Moreover, in a pregnant mouse model, the vertical transmission rate was high (87%) (Miller et al., 2002) with inocula lower than 2×10^6 tachyzoites per mouse, similar to the vertical transmission observed during infection with the Nc-Liv isolate using the same mouse model (91%) (Quinn et al., 2002). Significant *N. caninum*-specific antibody production was detected in the infected groups in both mouse models, with a dominant IgG1 response observed. The antibody levels varied with the isolate administered; inoculation of Nc-Spain1H induced a lower antibody response than inoculation of Nc-1. The smaller antigenic stimulus produced by the Nc-Spain1H isolate may be related to reduced parasite burden in mouse tissues. Variations in the immune responses of mice depending on the isolate inoculated have also been reported in a previous study, which suggested that this may be due to differences in the behavior of the isolates in a host (Quinn et al., 2002).

In the present study, we also used a BALB/c mouse model previously developed in our laboratory in which experimental infections with Nc-Liv and Nc-1 were characterized by an early phase of infection during which parasitemia and parasite DNA were observed in several murine organs, mainly the lungs, and by a chronic stage in which parasites were

detected in the brain (Collantes-Fernández et al., 2006). Results obtained from experimental Nc-Spain1H infections in mice demonstrated that this isolate failed to induce clinical signs or mortality and resulted in reduced burden, which was undetectable using our technique, or no parasite presence in the brain during the chronic stage, even in those mice inoculated with 10^7 tachyzoites. We also studied *N. caninum* antibody responses and showed that IgG2a and IgG1 isotype levels differed depending on the infectious dose administered. Mice inoculated with 10^5 tachyzoites showed a dominant IgG2a response, which might correlate with a cell-mediated response, that appears to limit the multiplication of the parasite. In addition, an increased inoculum size led to increased IgG1 levels. For the intracellular protozoa *Leishmania*, parasite load is suggested to affect the type of immune response developed; a high parasite load would favor a Th2 response, which directly downregulates Th1 cells (Hondowicz and Scott, 2002).

Our results suggest that Nc-Spain1H is a low virulence isolate with a low capacity for multiplication in host tissues and for transplacental transmission. However, the isolate was obtained from a congenitally infected calf, and it is likely that the parasite was transmitted from the mother to the fetus. Additional studies are necessary to investigate the pathogenicity of Nc-Spain1H in bovines.

This paper describes the successful isolation and characterization of an *N. caninum* isolate from an asymptomatic naturally infected calf, and its low virulence in BALB/c mice. This work may lead to further studies regarding the biological diversity among isolates and their association with the presentation of disease in cattle. Furthermore, it could constitute a successful approach for the identification of low virulence *N. caninum* isolates for use as live vaccine candidates.

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611 Table 1.

612 Assignment of alleles and the allelic profile obtained for the Nc-Spain1H isolate.

Nc-Spain1H													
Microsatellite loci	MS1A	MS1B	MS2	MS3	MS4	MS5	MS6A	MS6B	MS7	MS8	MS10	MS12	MS21
Allele no.	1	1	3	5	4	1	6	2	3	4	4	2	1
Repeat length	$(TA)_{60}$	$(AT)_{12}$	$(AT)_{23}$	$(AT)_{11}$	$(AT)_{18}$	$(TA)_{11}$	$(TA)_{18}$	$(AT)_{12}$	$(TA)_{14}$	$(AT)_{16}$	$(ACT)_{6}$-$(AGA)_{21}$-$(TGA)_{10}$	$(GT)_{16}$	$(TACA)_{10}$
GenBank accession no.	CS693095	CS693096	CS693097	CS693098	CS693099	CS693100	CS693101*		CS693102	CS693103	CS693104	CS693105	CS693106

613

614 * MS6A and MS6B are included in one sequence since they are both found at a unique locus.

615

Table 2.

Pregnancy rate, litter size, stillbirth, and vertical transmission in pregnant BALB/c mice inoculated with 2×10^6 Nc-Spain 1H or Nc-1 tachyzoites, or PBS buffer.

	Nc-Spain 1H	**Nc-1**	**PBS**
Pregnancy rate[a]	6/26 (23.1%)	12/27 (44.4%)	10/27 (37%)
Litter size[b]	4.2 ±1.1	5.4±1.7	5±1.6
Stillborn [c]	5/25 (20%)	14/65 (21.5%)	2/45 (4.4%)
Vertical transmission[d]	1/20 (5%)	39/42 (92.8%)	0/43 (0%)

[a] Number of pregnant mice/total number of mated mice (percentage).

[b] Average ± SD.

[c] Number of full-term dead pups at birth/total number of pups born (percentage).

[d] Number of positive PCR pups/total number of pups tested (percentage). Vertical transmission was calculated for the samples analyzed, as some samples could not be collected due to the cannibalism of dams.

Table 3.

Detection of *N. caninum* DNA by nested-PCR in blood, lung, and brain samples from BALB/c mice inoculated with 10^5, 10^6, or 10^7 Nc-Spain 1H tachyzoites.

Days p.i.	Blood [a]			Lung [a]			Brain [a]		
	10^5	10^6	10^7	10^5	10^6	10^7	10^5	10^6	10^7
1	1/3	3/3	4/4	0/3	0/3	3/4	1/3	1/3	1/4
2	1/3	1/3	4/4	0/3	3/3	4/4	1/3	1/3	3/4
4	1/3	1/3	4/4	0/3	3/3	4/4	1/3	2/3	3/4
8	0/3	0/3	0/3	1/3	1/3	3/3	0/3	0/3	1/3
16	0/3	0/3	0/3	0/3	1/3	0/3	0/3	0/3	0/3
32	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3

[a] Fractions represent the number of positive mice/total number of mice tested by nested-PCR.

Figure captions.

Fig. 1. Box-plot graph in figure A presents the lower and upper quartiles and the median yield of tachyzoites in cell cultures from two independent experiments performed in triplicate. In figure B, the bars represent the mean percentage of PV expressing BAG1 for Nc-Spain1H and Nc-Liv isolates from days 3 to 6.5 after stress, and error bars indicate the SD. Values correspond to data from two independent experiments performed in triplicate.

Fig. 2. Body weight of neonates born to dams inoculated with Nc-Spain1H or Nc-1 tachyzoites, or PBS buffer. Each point represents the mean body weight of all animals in each group on days 14, 16, 18, and 20 PP.

Fig. 3. Kaplan–Meier survival curves for neonates born to dams inoculated with Nc-Spain 1H or Nc-1 tachyzoites, or PBS buffer. The curves represent the percentage of animals surviving over a period of 30 days post-partum. Vertical steps downward correspond to days PP when a mouse was found death or was sacrificed. Symbols (■, ■, ▲) indicate censored observations. The number of dead mice was registered daily, and the percent survival of the Nc-Spain1H-infected group and uninfected mice was significantly greater than that of the Nc-1-infected group ($P<0.001$, Log-rank test).

Fig. 4. Bars represent the mean absorbance, and error bars indicate the SD of anti-*N. caninum* IgG1 and IgG2a isotypes from pregnant BALB/c mice (A) and non-pregnant BALB/c mice (B) injected with Nc-Spain1H, Nc-1 isolates, or PBS buffer. IgG1 and IgG2a antibody responses that were significantly different ($P< 0.0001$, ANOVA test) compared to those for the non-infected group are denoted *.

Fig. 5. Bars represent the mean absorbance, and error bars indicate the SD of anti-*N. caninum* IgG1 (A) and IgG2a (B) isotypes and ratio of IgG1/IgG2a (C) in BALB/c mice inoculated with 10^5, 10^6, or 10^7 Nc-Spain1H tachyzoites or PBS buffer. In graphs A and B, significant differences ($P< 0.0001$, ANOVA test) in antibody levels compared with the non-infected group are denoted *. Data corresponding to days 1, 2, and 4 p.i. are not represented because antibody responses developed by infected groups were not significantly different to those developed by control mice.

Figure 1

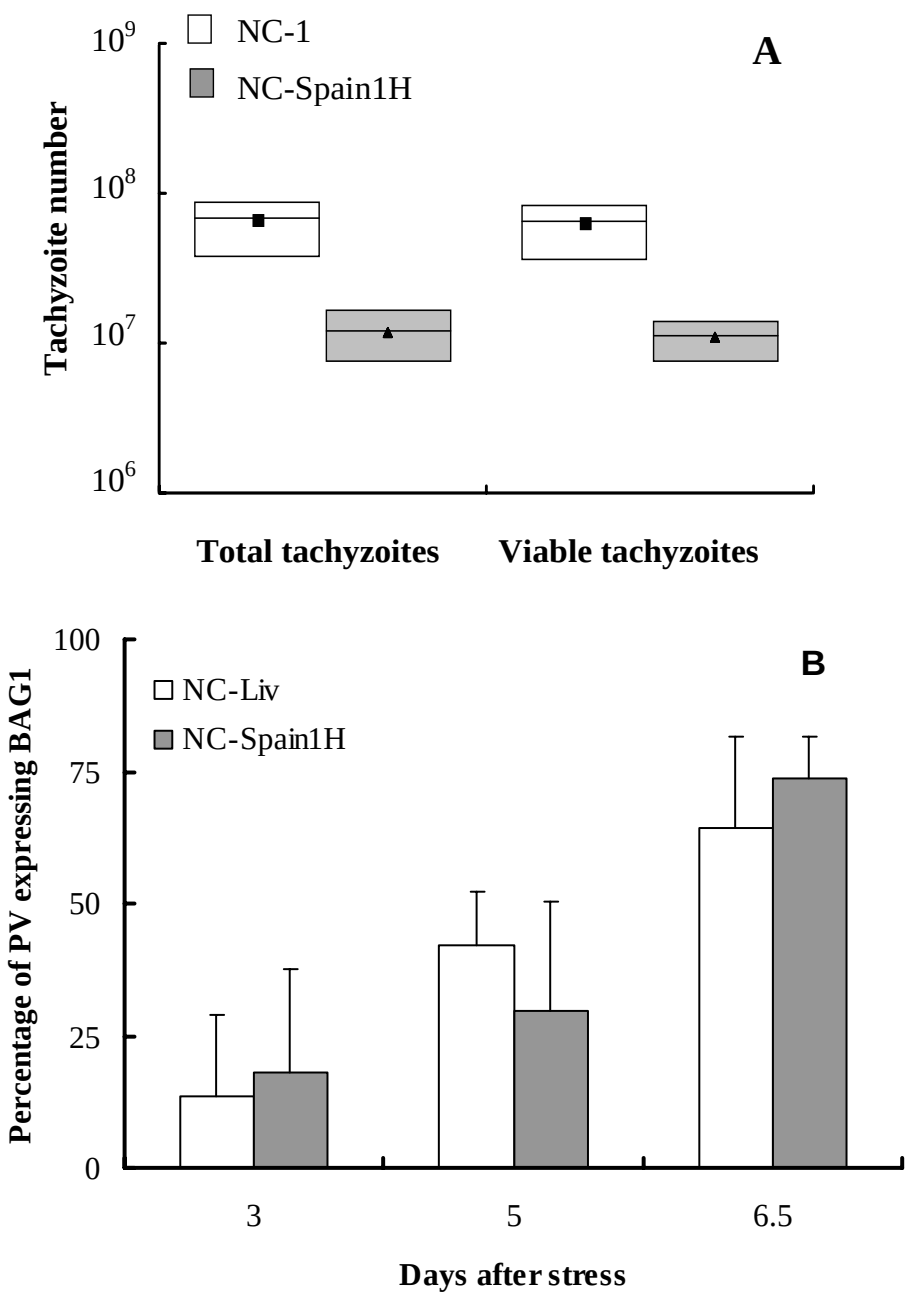

A
NC-1
NC-Spain1H
Tachyzoite number
10^9
10^8
10^7
10^6
Total tachyzoites
Viable tachyzoites
B
NC-Liv
NC-Spain1H
Percentage of PV expressing BAG1
100
75
50
25
0
3
5
6.5
Days after stress

Figure 2

1

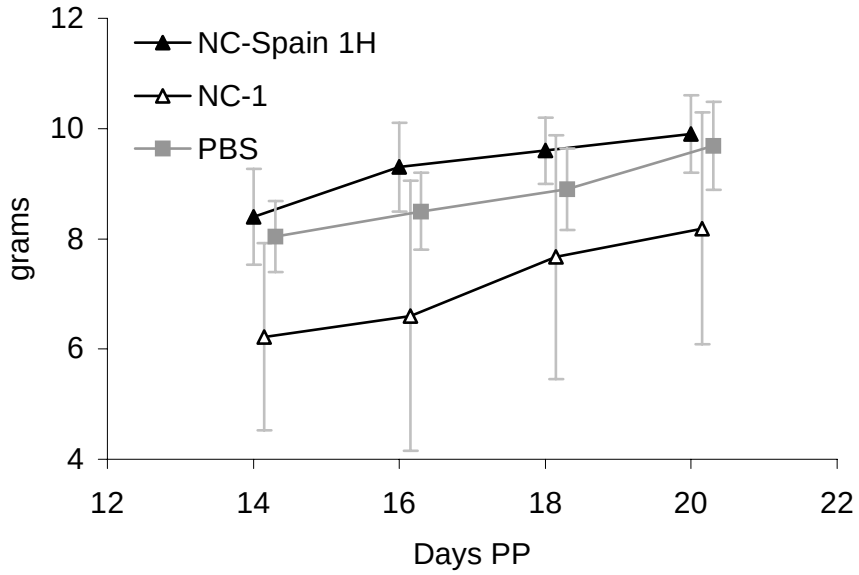


Figure 3

1

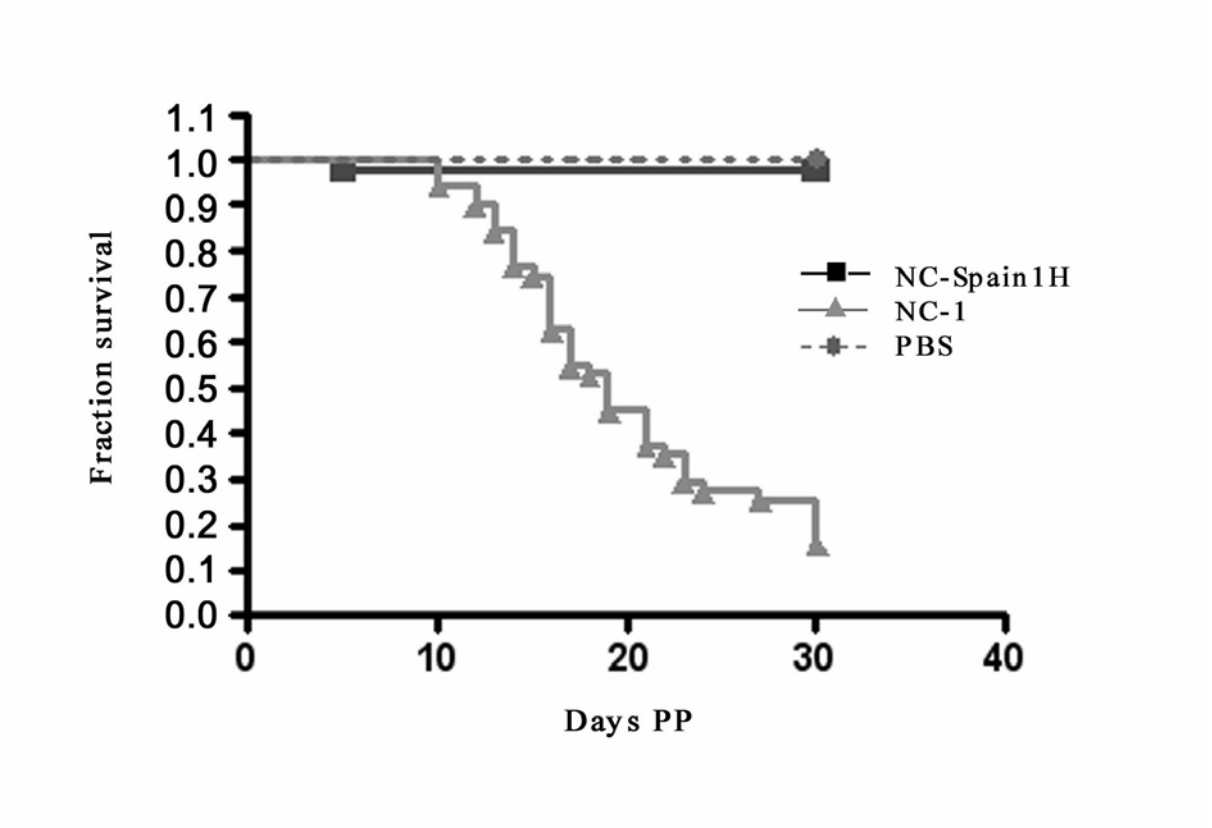

Fraction survival
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
10
20
30
40
Days PP
NC-Spain1H
NC-1
PBS

Figure 4

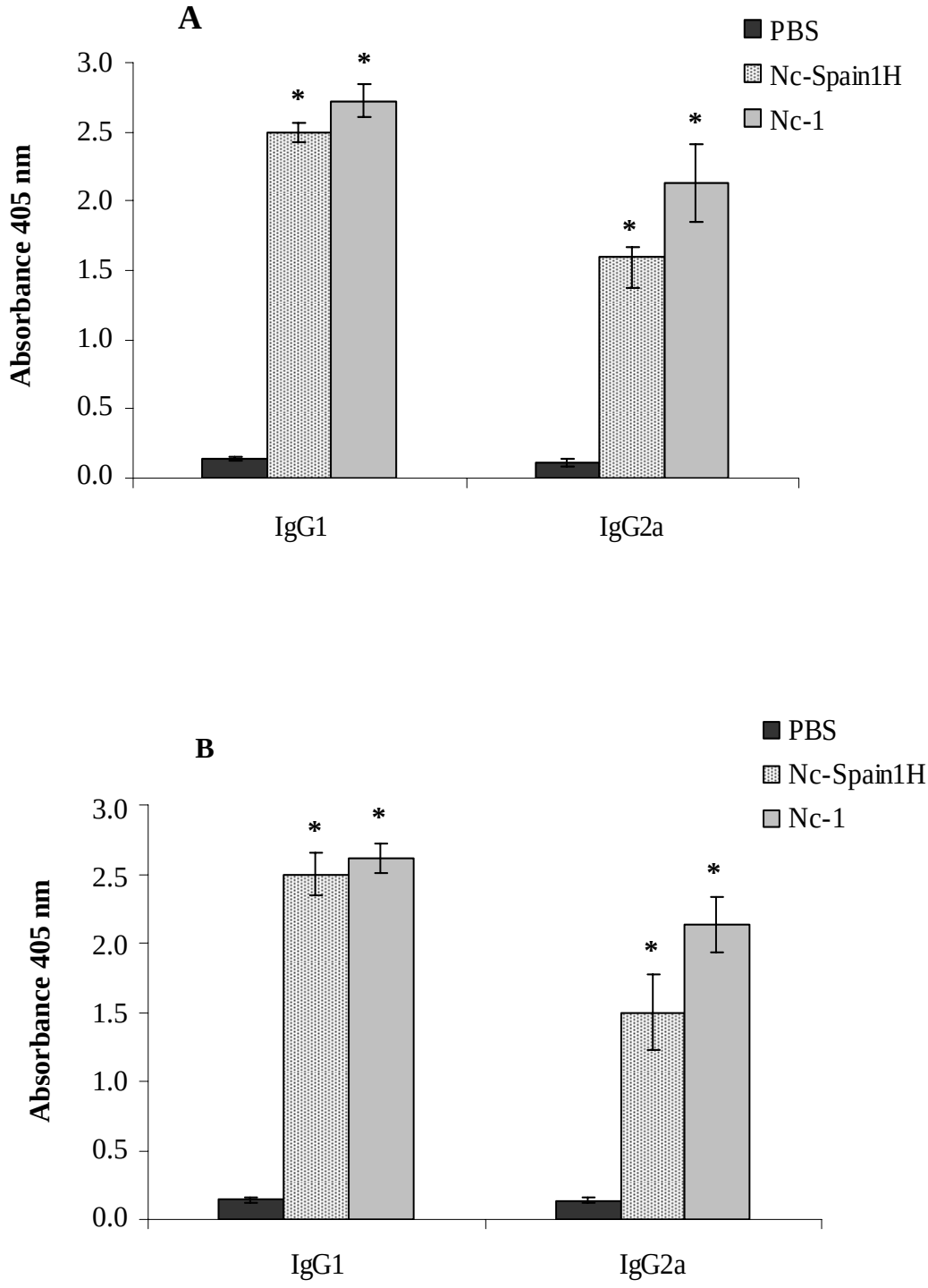
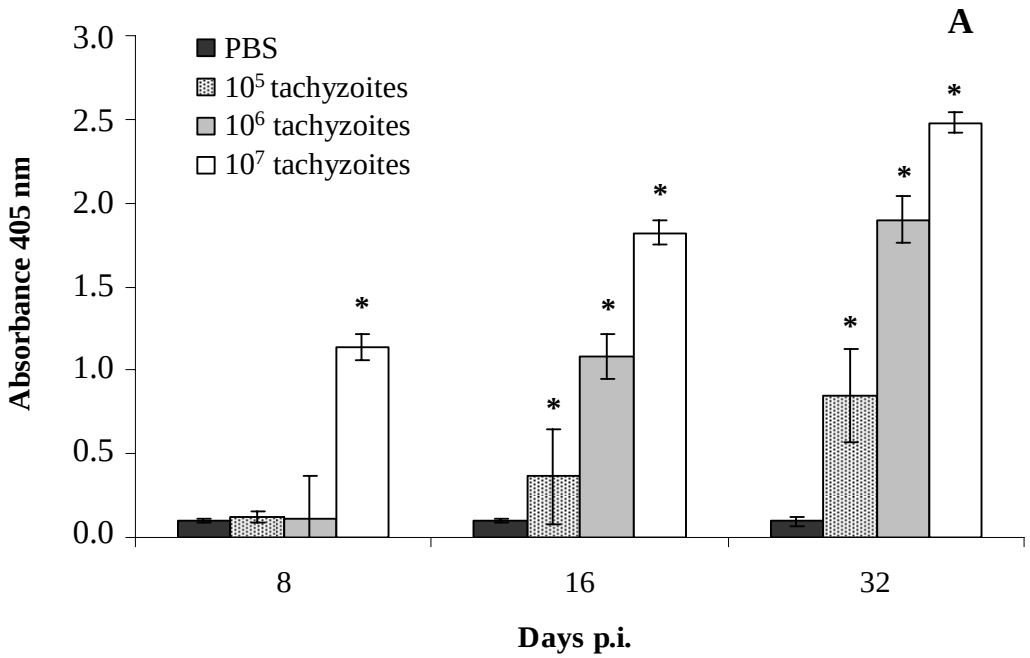
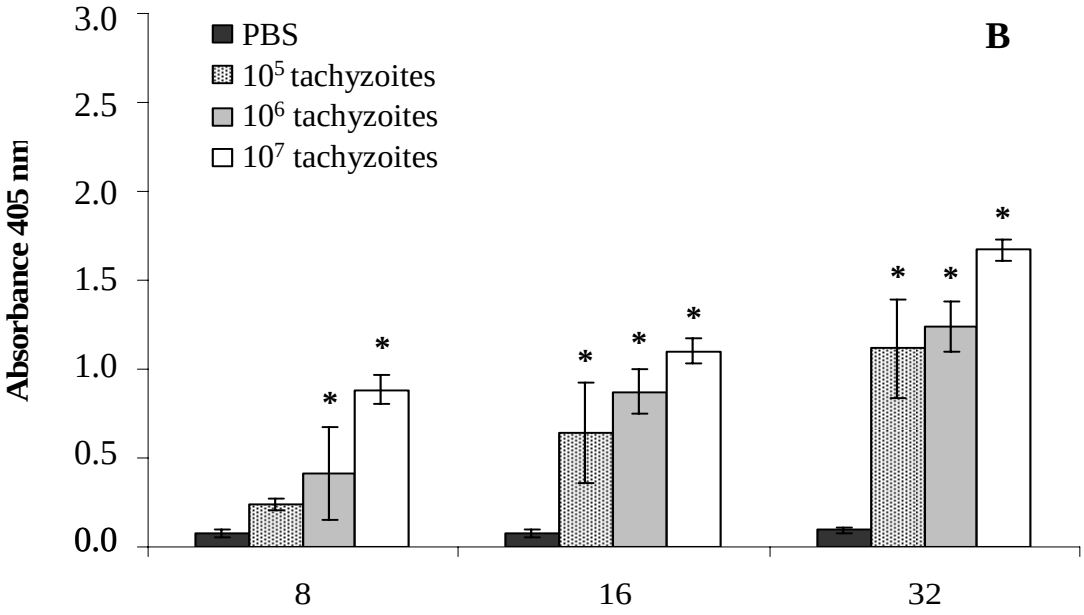
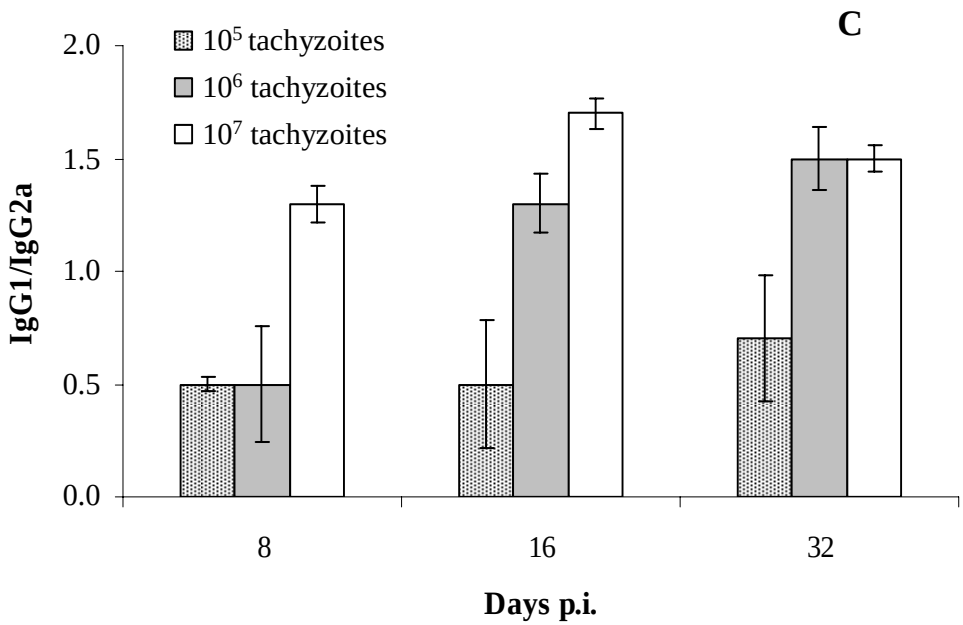

A
PBS
Nc-Spain1H
Nc-1
Absorbance 405 nm
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
IgG1
IgG2a
B
PBS
Nc-Spain1H
Nc-1
Absorbance 405 nm
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
IgG1
IgG2a

Figure 5


A
PBS
10^5 tachyzoites
10^6 tachyzoites
10^7 tachyzoites
Absorbance 405 nm
3.0
2.5
2.0
1.5
1.0
0.5
0.0
8
16
32
Days p.i.


B
PBS
10^5 tachyzoites
10^6 tachyzoites
10^7 tachyzoites
Absorbance 405 nm
3.0
2.5
2.0
1.5
1.0
0.5
0.0
8
16
32


C
10^5 tachyzoites
10^6 tachyzoites
10^7 tachyzoites
IgG1/IgG2a
2.0
1.5
1.0
0.5
0.0
8
16
32
Days p.i.