Specific antibody responses against *Neospora caninum* recombinant rNcGRA7, rNcSAG4, rNcBSR4 and rNcSRS9 proteins are correlated with virulence in mice

ELENA JIMÉNEZ-RUIZ†, GREGORI BECH-SÀBAT†, GEMA ÁLVAREZ-GARCÍA*, JAVIER REGIDOR-CERRILLO, LAURA HINOJAL-CAMPAÑA *and* LUIS M. ORTEGA-MORA

SALUVET, Animal Health Department, Faculty of Veterinary Sciences, Complutense University of Madrid, Ciudad Universitaria s/n, 28040 Madrid, Spain

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SUMMARY

The intraspecific diversity of *Neospora caninum* is a determinant for *in vivo* parasite virulence and *in vitro* parasite behaviour. The relationship between isolate virulence and specific antibody responses against key parasite proteins has not been well characterized. The response kinetics and the differences in specific anti-rNcGRA7, -rNcSAG4, -rNcBSR4 and -rNcSRS9 antibody levels were analysed by recombinant protein-based ELISA in groups of mice inoculated with 10 different *N. caninum* isolates that differ in their virulence. The majority of the virulence parameters analysed correlated with the specific antibody levels against the 4 recombinant proteins. The antibodies developed against the highly immunogenic protein NcGRA7 were significantly higher in mice inoculated with high virulence isolates than in those inoculated with low-to-moderate virulence isolates in both non-pregnant and pregnant mouse models. Moreover, these levels were correlated with the anti-*N. caninum* IgG1 and IgG2a responses and the *in vitro* tachyzoite yield at 56 h. The antibodies directed against the bradyzoite-specific proteins were not detected in a non-pregnant mouse model. However, some seropositive mice were found in groups inoculated with high virulence isolates in a pregnant mouse model. NcGRA7 and NcSAG4 are proteins clearly correlated with virulence, and to a lesser extent NcBSR4 and NcSRS9 proteins. Moreover, antibodies to bradyzoite-specific proteins appear to also be related to virulence in mice. Further analyses should be performed in order to verify the usefulness of these proteins as predictive markers for virulence in an experimental bovine model of neosporosis.

Key words: *Neospora caninum*, virulence, BALB/c mice, rNcGRA7, rNcSAG4, rNcBSR4, rNcSRS9, antibody response.

INTRODUCTION

Neospora caninum is a tissue cyst-forming coccidian parasite closely related to *Toxoplasma gondii* that causes reproductive failure in cattle worldwide (Dubey *et al*. 2007; Dubey and Schares, 2011). The pathogenesis of bovine neosporosis depends on several factors related to the host (e.g. infection route, immune responses of the pregnant cow and fetus and stage of gestation) and factors related to the parasite (parasite stage, dosage and isolate) (Dubey *et al*. 2006). In particular, it has recently been shown that *N. caninum* intraspecific diversity is a determinant of parasite virulence and *in vitro* parasite behaviour. Intra-specific diversity was first evidenced by genetic differences found among *N. caninum* isolates (Schock *et al*. 2001; Regidor-Cerrillo *et al*. 2006; Al-Qassab *et al*. 2009; Basso *et al*. 2009; Pedraza-Díaz *et al*. 2009). Later, several authors reported the influence of the *N. caninum* isolate used on the outcomes of infection and the immune responses to the infection in murine and bovine experimental models (Miller *et al*. 2002; Quinn *et al*. 2002; Collantes-Fernández *et al*. 2006; Rojo-Montejo *et al*. 2009; Pereira García-Melo *et al*. 2010; Regidor-Cerrillo *et al*. 2010; Caspe *et al*. 2012). Indeed higher anti-*N. caninum* IgG1-IgG2a humoral immune responses were induced by high virulence isolates than by isolates with low-to-moderate virulence (Pereira García-Melo *et al*. 2010; Regidor-Cerrillo *et al*. 2010). However, the host- and parasite-dependent mechanisms responsible for these differences have not been determined although several proteins (NcMIC1, NcNTPase, NcROP40, aspartyl tRNA synthetase and G6PD) have been identified as being more abundant in highly virulent isolates (Regidor-Cerrillo *et al*. 2012). One approach to determine the mechanisms behind the differing immune response is to investigate the proteins involved in the tachyzoite lytic cycle because high virulence isolates have displayed significantly higher invasion and proliferation rates in *in vitro* assays (Regidor-Cerrillo *et al*. 2011). However, bradyzoite-specific proteins are also interesting candidates because these proteins seem to be involved in the evasion of host immune response and persistence within the host tissues by *T. gondii* (Kim and Boothroyd, 2005; Kim *et al*. 2007). Interestingly, cystogenic isolates such as Nc-Liverpool and Nc-Spain 7 have proven to be virulent (Collantes-Fernández *et al*. 2006; Pereira García-Melo *et al*. 2010; Regidor-Cerrillo

* Corresponding author: SALUVET, Animal Health Department, Faculty of Veterinary Sciences, Complutense University of Madrid, Ciudad Universitaria s/n. 28040-Madrid, Spain. Tel: +34 913944095. Fax: +34 913944098. E-mail: gemaga@vet.ucm.es

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et al. 2010). In this sense, 4 proteins (NcGRA7, NcSAG4, NcBSR4 and NcSRS9) that are considered likely to be related to these 2 important processes (lytic cycle and persistence) were chosen in order to accomplish this work.

NcGRA7 is a dense granule protein involved in invasion and parasite proliferation that is expressed in both the tachyzoite and bradyzoite stages (Hemphill *et al*. 1998; Álvarez-García *et al*. 2007; Aguado-Martínez *et al*. 2010). NcSAG4 is an early-expressed bradyzoite-specific protein, and NcBSR4 and NcSRS9 are late-expressed bradyzoite-specific proteins (Fernández-García *et al*. 2006; Risco-Castillo *et al*. 2007, 2011).

The aim of the present study was to investigate the relationship between the specific humoral immune responses directed against rNcGRA7, rNcSAG4, rNcBSR4 and rNcSRS9 and the virulence of the parasite. To this end, differences in the kinetics and specific antibody levels against these proteins were analysed in mice inoculated with 10 *N. caninum* isolates, which differ in their virulence. Additionally, the association of these differences with *in vivo* and *in vitro* parameters was investigated.

MATERIALS AND METHODS

Parasites

The *N. caninum* isolates used in this study are shown in Table 1 and were classified into 2 groups (low-to-moderate and high virulence isolates) according to their *in vivo* and *in vitro* behaviour described in previous works (Pereira García-Melo *et al*. 2010; Regidor-Cerrillo *et al*. 2010, 2011). These isolates were maintained in monolayer cultures of the monkey kidney cell line MARC-145, as previously described (Pérez-Zaballos *et al*. 2005). All isolates used to infect mice were synchronized and tachyzoites were recovered from 3-day growth cultures when the greater part of them was still intracellular (at least 80% of undisrupted parasite vacuoles in the cell monolayer in order to maintain parasite viability). Moreover, isolates were collected when they were in a similar low number of passages with the purpose of minimizing its potential changes in virulence (Bartley *et al*. 2006). Tachyzoite number was determined by Trypan blue exclusion followed by counting in a Neubauer chamber, and the parasites were re-suspended in PBS at the required dose in a final volume of 200 μl per mouse. Mice were inoculated within 1 h of tachyzoite collection.

Mice and experimental design

Seven-week-old female BALB/c mice were obtained from a commercial supplier (Harlan Interfauna Ibérica, Barcelona, Spain). They were free of common viral, parasite and bacterial pathogens according to the results of the routine screening procedures performed by the manufacturer. The mice were fed *ad libitum* in a controlled environment with cycles of 12 h of light and 12 h of dark. All of the protocols involving animals were approved by the Animal Research Committee of Complutense University, Madrid, Spain, following the proceedings described by the Regulation of Internal Regime for Animal Research Committee (published at BOUC, no. 2, on 9 February 2006) and EU legislation (Council Directive 86/609/EEC).

Inoculations of 10 *N. caninum* isolates in non-pregnant and pregnant BALB/c mouse models of neosporosis have been published by Pereira García-Melo *et al*. (2010) and Regidor-Cerrillo *et al*. (2010). Briefly, the mice in both experimental models were examined daily for clinical signs indicative of neosporosis (rough hair coats, inactivity, anorexia and neurological signs consisting of head tilting, walking in circles, ataxia, pelvic limb weakness and paralysis). Severe, clinically affected mice were sacrificed by CO_2 inhalation.

In the non-pregnant mouse model, the mice were inoculated with sterile PBS (control group) or with 10^6 tachyzoites per mouse by an intra-peritoneal route (Table 1). Five mice from the *N. caninum*-inoculated groups and 3 mice from the control group were randomly sacrificed by CO_2 inhalation on 1, 2, 4, 8, 16, 32 and 64 days post-inoculation (p.i.) except for the Nc-Spain 5H-infected group, for which the 64 day p.i. time-point was not performed.

For the pregnant mouse model, the mice in all of the groups were subcutaneously (s.c.) inoculated with 2×10^6 tachyzoites of different *N. caninum* isolates (Table 1) or with sterile PBS (control group) by the subcutaneous route at day 7 of pregnancy. The neonates were housed with dams until day 30 post-partum (p.p.) when both neonates and dams were sacrificed by CO_2 inhalation. Blood samples from non-pregnant and pregnant mice were collected by cardiac puncture at necropsy. Sera were aliquoted and preserved at −80 °C for analysis by ELISA.

Cloning and purification of recombinant proteins

cDNAs used for cloning NcGRA7, NcSAG4, NcBSR4 and NcSRS9 were obtained from Nc-Liverpool isolate. In previous studies, no differences in nucleotide sequence were observed among isolates (Walsh *et al*. 2001; Fernández-García *et al*. 2006; Risco-Castillo *et al*. 2007, 2011). Both the rNcGRA7 and rNcSAG4 proteins were produced in the pET-45b (+) (Novagen, Germany) prokaryotic expression vector system following previously described procedures, with a few modifications (Fernández-García *et al*. 2006, Álvarez-García *et al*. 2007, Jiménez-Ruiz *et al*. 2012). rNcBSR4 and rNcSRS9 were cloned into the prokaryotic expression vector pRSET-C (Invitrogen, USA) as previously described (Risco-Castillo *et al*. 2007, 2011). After the bacteria were

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Table 1. *In vivo* and *in vitro* potential virulence-related parameters measured in 10 *Neospora caninum* isolates and their consensus classifications according to virulence

(The isolates were categorized into low, moderate or high virulence categories for each trait based on the significant differences found in previous studies for the different *in vivo* (Pereira García-Melo *et al*. 2010; Regidor-Cerrillo *et al*. 2010) and *in vitro* (Regidor-Cerrillo *et al*. 2011) parameters studied. Thus, the isolates that showed significantly higher or lower results for each parameter were considered as high and low virulence isolates, respectively. On the contrary, isolates that did not show significant differences from the other isolates were considered to be moderate virulence isolates. The consensus classification was as follows: high virulence isolates (isolates with 6 parameters considered as high and a maximum of 1 parameter considered as low) and low-to-moderate virulence isolates (the remaining isolates with less than 4 high parameters and more than 3 low parameters).)

	In vivo Non-pregnant mouse model		*In vivo* Pregnant mouse model					*In vitro*			
Isolates	Parasite presence. Early phase[a]	Parasite presence. Chronic phase[b]	Dam morbidity[c]	Parasite presence in dams[d]	Neonatal morbidity[e]	Neonatal mortality[f]	Vertical transmission rate[g]	IR_{4h}[h]	Td[i]	TY_{56h}[j]	Virulence (*consensus*)
Nc-Spain 2H	Moderate (85)	Low (20)	Low (0)	Low (27·2)	Moderate (46·1)	Moderate (20·4)	Low (61·3)	Moderate (3726)	Moderate (11·7)	Low (5328)	Low-to-moderate
Nc-Spain 3H	High (95)	Low (13·3)	Low (0)	Moderate (78·5)	Low (10·6)	Low (7·7)	High (89)	Low (1088)	Moderate (12·1)	Low (5163)	Low-to-moderate
Nc-Spain 4H	Moderate (80)	Moderate (33·3)	High (100)	High (91·6)	High (100)	High (100)	High (97·3)	High (8758)	Low (14·2)	High (35500)	High
Nc-Spain 5H	Low (40)	High (80)	High (27·7)	High (100)	High (98·6)	High (96)	High (100)	Moderate (1657)	High (10·3)	High (18030)	High
Nc-Spain 6	High (90)	Low (23·3)	Low (0)	Low (25)	Moderate (34·5)	Moderate (29·8)	Low (57·6)	Moderate (976·5)	High (9·8)	High (17110)	Low-to-moderate
Nc-Spain 7	Low (30)	High (60)	High (30·7)	Moderate (69·2)	High (98·3)	High (95)	High (79·1)	Moderate (5249)	Moderate (11·6)	High (36170)	High
Nc-Spain 8	Moderate (65)	Moderate (30)	Low (0)	Low (26·3)	Low (4·7)	Low (1·1)	Low (56·4)	Moderate (2991)	Moderate (13·3)	Low (1731)	Low-to-moderate
Nc-Spain 9	Moderate (80)	High (63·3)	Low (0)	Low (10)	Moderate (39)	Moderate (32·5)	Low (52·6)	Moderate (1911)	Moderate (12·8)	Moderate (11140)	Low-to-moderate
Nc-Spain 10	N.D.	N.D.	Low (0)	Moderate (84·6)	Moderate (25·5–20·9)	Moderate (17·9)	Moderate (65·5)	Moderate (1729)	Moderate (13·45)	High (22190)	Low-to-moderate
Nc-Liverpool	N.D.	N.D.	High (62·5)	High (100)	High (100)	High (100)	High (95·6)	High (5325)	High (10·78)	High (16270)	High

a Parasite presence in the lungs during the early phase of *N. caninum* infection (1–8 days p.i.; average percentages).
b Parasite presence in the brain during the chronic phase of *N. caninum* infection (16–64 days p.i.; average percentages).
c No. of dams with clinical signs/no. of dams (percentage).
d No. of nested-PCR positive dams at day 30 p.p./no. of dams in the group (percentage).
e No. of pups with clinical signs from day 3 to 30 p.p./no. of pups (percentage).
f No. of pups dead from day 3 to 30 p.p./no. of pups (percentage).
g No. of nested-PCR positive pups/no. of pups per group (percentage).
h Invasion rate measured as the median number of intracellular parasites/scan area at 4 hours post-infection (average).
i Doubling time (average hours).
j The tachyzoite yield was defined as the average value of the number of tachyzoites quantified by qPCR at 56 h p.i.
N.D., not determined.

lysed, rNcGRA7 was purified from the soluble fraction by immobilized metal ion-affinity chromatography (IMAC) using HisTrapHP columns (GE Healthcare, USA) (Álvarez-García *et al*. 2007). rNcSAG4, rNcSRS9 and rNcBSR4 were extracted from inclusion bodies and denatured with a binding buffer containing 20 mM phosphate salts, 8 M urea and 40 mM imidazole (Aguado-Martínez *et al*. 2008). These recombinant proteins were obtained using an on-column refolding and purification procedure based on a method previously reported (Zhao *et al*. 2005).

The concentration and purity of the recombinant proteins were determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) with a standard BSA scale (Roche, Switzerland) and by Western blotting with a monoclonal anti-T7 tag antibody (Novagen, Germany) and polyclonal anti-rNcGRA7, rNcSAG4, rNcBSR4 and rNcSRS9 mouse sera (Fernández-García *et al*. 2006; Aguado-Martínez *et al*. 2008; Risco-Castillo *et al*. 2007, 2011). The protein concentrations were measured using Quantity One software (v. 7.2, Bio-Rad, USA). All of the proteins were stored at −80 °C until use.

Analysis of the humoral immune response

The ELISAs for detecting specific IgGs developed against rNcGRA7, rNcSAG4, rNcBSR4 and rNcSRS9 were carried out as described previously (Rojo-Montejo *et al*. 2011). Briefly, 96-well plates were coated with 0·1 μg/well of rNcGRA7 or rNcSAG4 and 0·2 μg/well of rNcBSR4 or rNcSRS9. The serum samples were applied at a 1:100 dilution and an anti-mouse IgG peroxidase-conjugated antibody (Sigma, USA) was used at a 1:3000 dilution. For the non-pregnant mouse model, only sera from 8, 16, 32 and 64 days p.i. were analysed because the production of specific antibodies against *N. caninum* and against rNcGRA7 occurs from 8 days p.i. onwards and the production of those against rNcSAG4 occurs from 14 days p.i. onwards (Collantes-Fernández *et al*. 2006; Aguado-Martínez *et al*. 2009*a*). Control group samples for each day were also analysed.

Sera from mice immunized with rNcSAG4, rNcGRA7, rNcBSR4 and rNcSRS9 in previous studies were employed as positive controls, whereas sera from non-immunized uninfected mice were used as negative controls in each plate (Aguado-Martínez *et al*. 2009*b*; Jiménez-Ruiz *et al*. 2012). Serum samples were analysed in duplicate. The threshold value (cut-off) was defined arbitrarily to discriminate between 'positive' and 'negative' by adding 2 standard deviations to the mean A405 value of sera from non-immunized uninfected mice. The cut-off values corresponded to 0·27, 0·29, 0·24 and 0·42 optical density (O.D.) values for the rNcGRA7, rNcSAG4, rNcBSR4 and rNcSRS9 ELISA tests, respectively.

The ELISAs for detecting specific IgG1 and IgG2a against *N. caninum* extract were performed by Pereira Garcia-Melo *et al*. (2010) and Regidor-Cerrillo *et al*. (2010). Briefly, an ELISA was developed with soluble *N. caninum* tachyzoite antigen (0·5 μg in 100 μl/well) using diluted serum samples (1:100) and anti-mouse IgG2a or IgG1 antibody (1:5000; Southern Biotechnology, Birmingham, AL, USA). Serum samples were analysed in duplicate.

Statistical analysis

A one-way ANOVA followed by Duncan's multiple range tests was employed to compare specific antibody levels (measured as optical density (O.D.) values) developed against the 4 recombinant proteins included in this study. In the non-pregnant model, comparisons were performed between groups inoculated with low-to-moderate *vs* high virulence isolates for each day evaluated (8, 16, 32, 64 days p.i.). Moreover, different day p.i. samples were also compared for each isolate. In the pregnant model, the specific antibody levels were compared between groups of mice inoculated with different isolates or grouped in virulence categories (low-to-moderate *vs* high virulence isolates). The significance for these analyses was established at $P < 0{\cdot}05$.

Spearman's rank correlation coefficient (ρ) was applied to investigate the potential association between the specific antibody levels and different parameters of isolate virulence reported in previous studies. In the non-pregnant mouse model, the percentages of mice with parasites present in the brain at 8, 16, 32 and 64 days p.i. for each inoculated group were compared with median O.D. values of specific antibodies developed against each recombinant protein (Pereira García-Melo *et al*. 2010). Because no morbidity and mortality rates were recorded, correlation analyses were not performed. In the pregnant mouse model, parameters related to virulence in dams (morbidity and parasite presence) and in pups (neonatal morbidity and mortality and vertical transmission rates) (Regidor-Cerrillo *et al*. 2010) were compared with the median values of the specific antibodies developed against the recombinant proteins. The O.D. values of IgG1 and IgG2a antibodies against the *N. caninum* soluble extract detected in the non-pregnant model at 8, 16, 32 and 64 days p.i. by Pereira García-Melo *et al*. (2010) were correlated with the corresponding individual values of specific antibodies against the recombinant proteins. Similar analyses were conducted for the pregnant model (Regidor-Cerrillo *et al*. 2010).

Additionally, *in vitro* parameters such as the invasion rate at 4 h (IR_{4h}), doubling time (Td) and

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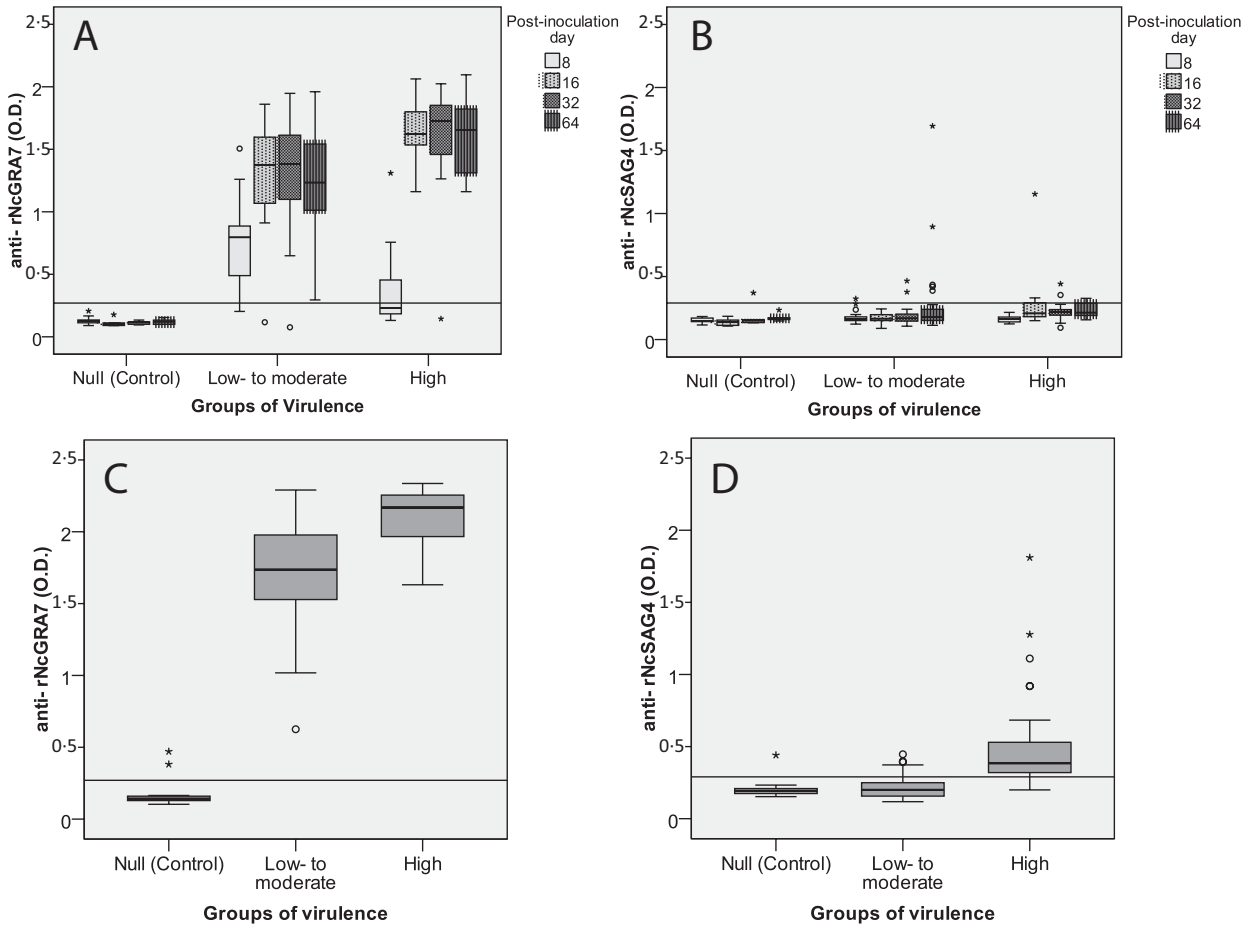

Fig. 1. Box-plot graphs representing the median, lower and upper quartiles (boxes), non-outlier minimum and maximum (whiskers), and outlier (°; values 1·5-fold more than the inter-quartile range away from the quartiles) and extreme (*; values 3-fold more than the inter-quartile range away from the quartiles) optical density (O.D.) values obtained with rNcGRA7 (A) and rNcSAG4 (B) from non-pregnant mice sera at different days post-infection and rNcGRA7 (C) and rNcSAG4 (D) from pregnant mice sera at day 30 post-partum. Mice were inoculated with isolates of low-to-moderate virulence (Nc-Spain 2H, Nc-Spain 3H, Nc–Spain 6, Nc-Spain 8 and Nc-Spain 9), high virulence (Nc-Spain 4H, Nc-Spain 5H, Nc-Spain 7) or with PBS (control group). The horizontal lines represent the cut-off point for each test.

tachyzoite yield at 56 h (TY_{56h}) obtained in another recent study (Regidor-Cerrillo *et al.* 2011) were compared with the median specific anti-rNcGRA7 antibody values detected in the non-pregnant and pregnant models for each isolate. In this analysis, the antibody levels against bradyzoite-specific proteins were not included because the *in vitro* assays were carried out exclusively with tachyzoites.

All statistical analyses were performed and graphics generated using IBM SPSS Statistics v.19 software.

RESULTS

Non-pregnant mouse model

All of the groups inoculated with the *N. caninum* isolates developed specific antibodies against rNcGRA7 (Fig. 1A). When the immune responses were analysed to compare days p.i. within each group, the anti-rNcGRA7 levels were higher during the chronic infection in all groups (16, 32 and 64 days p.i.) ($P<0·05$) compared with acute infection (8 days p.i.), but no differences were detected between the different days of chronic infection. Additionally, higher O.D. values of specific anti-*Neospora* IgG1 and IgG2a antibodies were correlated with higher specific anti-rNcGRA7 levels at each day p.i. analysed (Table 2).

When the specific anti-rNcGRA7 antibody levels were compared between the low-to-moderate and high virulence groups, significant differences were observed (Fig. 1A). During the early infection (8 days p.i.), the levels of anti-rNcGRA7 were higher in the low-to-moderate virulence groups ($P<0·05$). In this case, the isolates Nc-Spain 2H, 3H, 6 and 8 produced a significantly higher humoral immune response against rNcGRA7 than Nc-Spain 4H, 5H and 7 ($P<0·05$). On the contrary, during the late infection (16, 32 and 64 days p.i.), the highest anti-rNcGRA7 levels corresponded to the high virulence isolate group with significant differences between 16 and

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Table 2. Correlation analyses of specific antibody values against rNcGRA7, rNcSAG4, rNcBSR4 and rNcSRS9 with anti-IgG1 and IgG2a values and *in vivo* parameters measured in both the non-pregnant and pregnant mouse models

Model	Specific antibodies	Days	IgG1		IgG2a		Dams morbidity		Parasite presence in dams		Neonatal morbidity		Neonatal mortality		Vertical transmission	
			R^{2a}	P[b]	R^{2a}	P[b]	ρ[c]	P[b]	ρ[c]	P[b]	ρ[c]	P[b]	ρ[c]	P[b]	ρ[c]	P[b]
Non-pregnant[d] (Pereira García-Melo *et al.* 2010)	rNcGRA7	8 p.i.	0·317	0·038	0·739	<0·001	–	–	–	–	–	–	–	–	–	–
		16 p.i.	0·442	0·003	0·495	0·001	–	–	–	–	–	–	–	–	–	–
		32 p.i.	0·650	<0·001	0·557	<0·001	–	–	–	–	–	–	–	–	–	–
		64 p.i.	0·706	<0·001	0·618	<0·001	–	–	–	–	–	–	–	–	–	–
Pregnant (Regidor-Cerrillo *et al.* 2010)	rNcGRA7	30 p.p.	0·896	<0·001	0·904	<0·001	0·748	0·008	N.C.	–	0·815	0·002	0·752	0·008	0·609	0·047
	rNcSAG4	30 p.p.	N.C.	–	0·222	0·008	0·832	0·001	0·679	0·022	0·688	0·019	0·733	0·01	0·764	0·006
	rNcBSR4	30 p.p.	N.C.	–	0·160	0·061	0·695	0·018	0·62	0·042	0·679	0·022	0·724	0·012	0·600	0·051
	rNcSRS9	30 p.p.	N.C.	–	0·174	0·039	0·642	0·033	N.C.	–	N.C.	–	0·638	0·035	0·673	0·023

a R^2 of Pearson.
b P-value (two-tailed).
c Spearman rho coefficient.
d Morbidity and mortality rates were not recorded and correlation analyses were not performed.
N.C., no correlation.

64 days p.i ($P<0·05$). The Duncan post-hoc test identified the isolates that produced the lowest antibody response at 16 days p.i. (Nc-Spain 3H), 32 days p.i. (Nc-Spain 2H, 3H) and 64 days p.i. (Nc-Spain 2H, 3H and 8).

Regarding the bradyzoite stage-specific proteins, no antibody levels against rNcSAG4 (Fig. 1B), rNcBSR4 or rNcSRS9 (data not shown) were observed over the cut-off values for each test. Therefore, no further comparisons were performed for these proteins in the non-pregnant model.

The correlation analyses between parasite presence and specific antibody levels against rNcGRA7 were not significant at any period of time (data not shown). Moreover, significant correlations could be found between the average values of the *in vitro* parameter TY_{56h} and median values of specific antibodies against rNcGRA7 at 32 days p.i. and 64 days p.i. (Table 3).

All statistical analyses were also performed with normalized ELISA data measured as relative index per cent (R.I.P.C. = (O.D. sample − O.D. negative control)/(O.D. positive control − O.D. negative control) × 100). The results with R.I.P.C.s data showed similar significant variations to the results obtained with data not normalized but for TY_{56h} and specific antibodies against rNcGRA7 at 64 days p.i. (Supplementary Table 1 and Table 2, online version only).

Pregnant mouse model

All of the mice inoculated with any of the *N. caninum* isolates developed specific antibodies against rNcGRA7 above the cut-off value. The high virulence isolate group developed significantly higher specific antibody levels against rNcGRA7 than the low-to-moderate virulence group (Fig. 1C, $P<0·05$). In the case of rNcSAG4, 16·7% and 84·1% of the samples belonging to the low-to-moderate and high virulence groups, respectively, showed detectable specific antibody levels that were significantly higher in the high virulence group compared with the low-to-moderate virulence group (Fig. 1D, $P<0·05$). The antibody levels against the late-expressed bradyzoite stage-specific proteins rNcBSR4 and rNcSRS9 were close to the cut-off values (data not shown). However, specific antibody levels above the cut-off were detected in 6·4% and 47·6% of mice for rNcBSR4 ($P<0·0001$; Fisher's exact test) and 3·8% and 37·2% for rNcSRS9 ($P<0·0001$; Fisher's exact test) in the low-to-moderate and high virulence isolate groups, respectively.

Higher levels of anti-rNcGRA7 antibodies were detected in the mice that were inoculated with the Nc-Spain 4H, Nc-Spain 5H and Nc-Spain 7 isolates ($P<0·05$) (Fig. 2A). Regarding rNcSAG4, dams inoculated with the high virulence isolates (Nc-Spain 4H, Nc-Spain 5H, Nc-Spain 7 and Nc-Liverpool)

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Table 3. Spearman correlation analyses of median values of specific antibodies against rNcGRA7 and the *in vitro* parameters for each isolate

		Median IR^{a}_{4h}		Average Td[a]		TY^{a}_{56h}	
Model	Days p.i.	ρ*	P#	ρ*	P#	ρ*	P#
Non- pregnant (Pereira García-Melo *et al*. 2010)	8	N.C.	–	N.C.	–	N.C.	–
	16	N.C.	–	N.C.	–	N.C.	–
	32	N.C.	–	N.C.	–	0·810	0·015
	64	N.C.	–	N.C.	–	0·821	0·023
Pregnant (Regidor-Cerrillo *et al*. 2010)		0·673	0·033	N.C.	–	N.C.	–

a *In vitro* parameters reported by Regidor-Cerrillo *et al*. 2011.
IR_{4h}, invasion rate at 4 h.
Td, doubling time.
TY_{56h}, tachyzoite yield at 56 h.
* Spearman rho coefficient.
# P-value (two-tailed).
N.C., no correlation.

developed specific antibodies above the cut-off point, whereas the dams inoculated with the low-to-moderate virulence isolates (Nc-Spain 2H, Nc-Spain 3H, Nc-Spain 8 and Nc-Spain 9; Fig. 2B) remained seronegative ($P<0·05$). Only some mice from the groups inoculated with Nc-Spain 4H (50%), Nc-Spain 5H (70·6%), Nc-Spain 6 (25%) and Nc-Liverpool (37·6%) showed a specific response against rNcBSR4 above the cut-off value (Fig. 2C). Additionally, some mice inoculated with the high virulence isolates, such as Nc-Spain 4H (80%), Nc-Spain 5H (29·4%) and Nc-Spain 7 (37·5%), recognized rNcSRS9, but no rNcSRS9-specific antibodies were developed by mice inoculated with the low-to-moderate virulence isolates, such as Nc-Spain 2H, Nc- Spain 3H, Nc-Spain 8 and Nc-Spain 9 (Fig. 2D).

Moreover, the IgG1 and IgG2a individual values were highly correlated with the levels of specific antibodies against rNcGRA7 (Table 2). Almost all of the virulence parameters analysed correlated with the specific antibody levels against the 4 recombinant proteins ($\rho>0·6$; $P<0·05$; Table 2). Additionally, regarding the *in vitro* parameters, the median IR_{4h} could be correlated with the median specific antibody levels against rNcGRA7 ($\rho=0·673$; $P=0·033$). There was no correlation between any of the specific antibody responses analysed and the other *in vitro* parameters (Table 3).

All statistical analyses were also performed with normalized ELISA data measured as relative index per cent (R.I.P.C. = (O.D. sample − O.D. negative control)/(O.D. positive control − O.D. negative control) × 100). The results with R.I.P.C.s data showed similar significant variations to the results obtained with data not normalized except for a few exceptions: anti-rNcSAG4 antibody levels correlated with anti-*N. caninum* IgG1 antibody levels. In a similar way anti-rNcBSR4 antibody levels correlated with both anti-*N. caninum* IgG1 antibody levels and neonatal morbidity. On the other hand, anti-rNcBSR4 antibody levels did not correlate with parasite presence in dams. However, the ρ value was low in case of both late-expressed bradyzoite proteins (Supplementary Table 1, online version only). Moreover, the same variation was observed in the case of anti-rNcGRA7 antibody levels developed by pregnant mice *vs* IR_{4h} (Supplementary Table 2, online version only).

DISCUSSION

The specific antibody responses against proteins involved in the lytic cycle (NcGRA7) and the tachyzoite-bradyzoite transition (NcSAG4, NcBSR4 and NcSRS9) were investigated for the first time and were shown to be correlated with virulence in mice infected with *N. caninum*. Differences in virulence-related parameters such as morbidity, mortality and abortions between different *N. caninum* isolates have been observed in previous *in vivo* experiments (Miller *et al*. 2002; Quinn *et al*. 2002; Collantes-Fernández *et al*. 2006; Rojo-Montejo *et al*. 2009; Regidor-Cerrillo *et al*. 2010; Pereira García-Melo *et al*. 2010; Caspe *et al*. 2012). These findings correlate with specific antibody responses because high virulence isolates (i.e. Nc-Spain 5H, 7 and Nc-Liverpool) induced higher levels of anti-*N. caninum* IgG1 and IgG2 than low-to-moderate virulence isolates (i.e.: Nc-Spain 3H, 2H and 9) (Regidor-Cerrillo *et al*. 2010). Interestingly, Regidor-Cerrillo *et al*. (2012) recently used a proteomics approach to show that some proteins are more abundant in highly virulent *N. caninum* isolates. However, *Neospora* virulence factors remain to be elucidated, and the host- and parasite-dependent mechanisms

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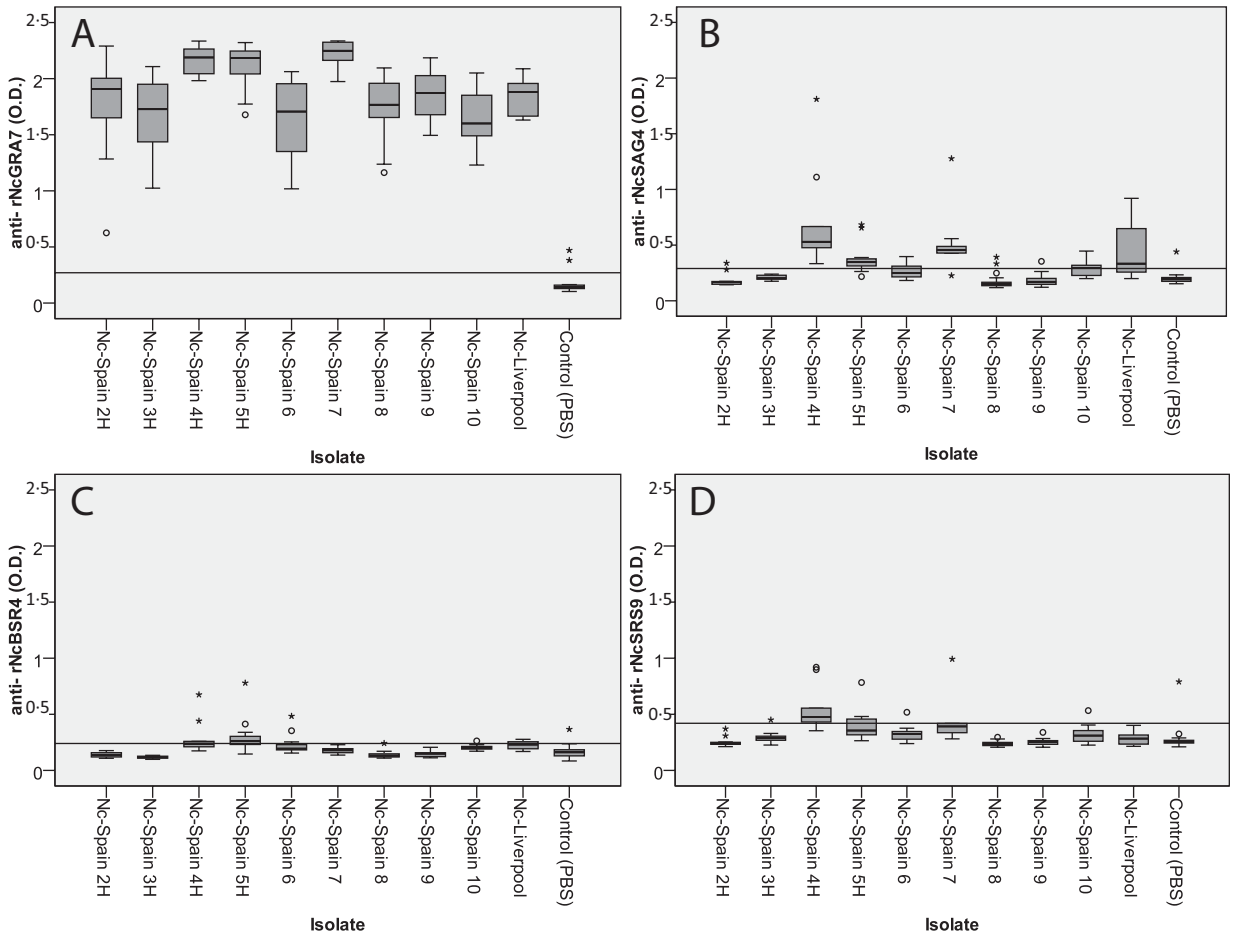

Fig. 2. Box-plot graphs representing the median, lower and upper quartiles (boxes), non-outlier minimum and maximum (whiskers), and outlier (°; values 1·5-fold more than the inter-quartile range away from the quartiles) and extreme (*; values 3-fold more than the inter-quartile range away from the quartiles) optical density (O.D.) values obtained with rNcGRA7 (A), rNcSAG4 (B), rNcBSR4 (C) and rNcSRS9 (D) antigen-based ELISAs from serum samples of mice inoculated with different isolates (Nc-Spain 2H (n=11), Nc-Spain 3H (n=14), Nc-Spain 6 (n=12), Nc-Spain 8 (n=19), Nc-Spain 9 (n=10) and Nc-Spain 10 (n=13) low-to-moderate virulence isolates- and Nc-Spain 4H (n=10), Nc-Spain 5H (n=17), Nc-Spain 7 (n=9) and Nc-Liv (n=8) high virulence-) or with PBS (control group) in the pregnant mouse model. The samples were taken at day 30 post-partum from mice inoculated at day 7 of gestation. The horizontal lines represent the cut-off point for each test.

responsible for the differences observed in the humoral immune responses developed by mice inoculated with isolates that differ in virulence have not been identified.

When the specific antibody levels against rNcGRA7, rNcSAG4, rNcBSR4 and rNcSRS9 developed by both pregnant and non-pregnant mice inoculated with 9 different bovine Spanish isolates and the Nc-Liverpool isolate were studied in the present work, significant correlations between the specific humoral immune responses developed against these proteins and several *in vivo* and *in vitro* parameters were observed.

The specific antibody responses developed against rNcGRA7 were correlated with virulence parameters in both non-pregnant and pregnant mouse models. It has been postulated that a higher parasite burden of the high virulence isolates increases the exposure of the mice to the protein and, consequently, a major production of specific anti-rNcGRA7 immunoglobulins (Aguado-Martínez *et al*. 2009*a*). However, the tendency of the high virulence isolates to produce higher levels of antibodies against rNcGRA7 was reversed in non-pregnant mice during the early infection (8 days p.i.) when low-to-moderate virulence isolates showed higher levels of anti-rNcGRA7 antibodies. This result is in accordance with the higher parasite burden observed in the blood and lungs of mice inoculated with low-to-moderate virulence isolates during the acute phase of infection reported by Pereira García-Melo *et al*. (2010). In this case, it is thought that the low-to-moderate virulence isolates could be more exposed to the host cellular and humoral immune responses during the parasitaemia period that is evidenced by higher levels

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of *N. caninum*-specific antibodies and may lead to a faster clearance of the parasite. On the other hand, the highest levels of anti-rNcGRA7 detected in sera from mice inoculated with high virulence isolates corresponded to the chronic phase of infection (16–64 days p.i.) in the non-pregnant model and day 30 p.p. in the pregnant model when the morbidity and mortality rates were recorded. Moreover, the pattern of recognition of rNcGRA7 observed in our study was in accordance with the results observed previously by Aguado-Martinez *et al*. (2009*a*), who reported higher levels of anti-rNcGRA7 antibodies in mice inoculated with the Nc-Liverpool isolate compared with mice inoculated with the Nc-1 isolate. However, virulence-related parameters such as dam morbidity, neonatal morbidity and mortality and vertical transmission were correlated with specific anti-rNcGRA7 antibody levels. In addition, a correlation between the *N. caninum*-specific IgG1 and IgG2a antibodies and the production of specific antibodies against rNcGRA7 was observed in both the non-pregnant and pregnant mouse models. This result is in accordance with previous mouse assays that reported high morbidity rates in mice when high anti-*N. caninum* IgG1 and IgG2a and anti-rNcGRA7 responses were detected in response to a high virulence isolate (Aguado-Martínez *et al*. 2009*a*). In this sense, higher levels of anti-rNcGRA7 antibodies induced by high virulence isolates could be explained by increases in the parasite dissemination and growth rate in host tissues compared with low-to-moderate virulence isolates because a correlation with the TY56 h values was detected. This hypothesis is supported by several different findings. It is known that the highly immunogenic dense granule protein NcGRA7 is involved in invasion and is associated with the active replication of the parasite as well as the establishment of the parasitophorous vacuole in the host cell (Hemphill *et al*. 1997; Aguado-Martínez *et al*. 2010). Moreover, in a recent study of *in vivo N. caninum* dissemination, low-to-moderate virulence isolates showed a lower efficiency of transmigration within dendritic cells and a lower gliding motility than high virulence isolates (Collantes-Fernandez *et al*. 2012). Therefore, the differences observed between the infected groups could reflect the different abilities of the isolates to replicate and disseminate within the host, in agreement with the correlations found between the *in vivo* virulence parameters and the *in vitro* parasite invasion and proliferation rates reported by Regidor-Cerrillo *et al*. (2011). However, the outcome of a *N. caninum* infection is affected by a combination of host and parasite factors, whereas *in vitro* parameters are only parasite dependent, so associations between the *in vitro* and *in vivo* behaviour of *N. caninum* isolates should be considered with caution.

Regarding the bradyzoite stage-specific proteins, no specific antibodies against the immunogenic protein rNcSAG4 in the non-pregnant mouse model were detected, contrary to the results obtained by Aguado-Martínez *et al*. (2009*a*); this difference might be due to a lower parasite dose employed since a dose of 10^6 tachyzoites per mouse was used in our study *vs* a dose of 2×10^6 tachyzoites per mouse used by Aguado-Martínez *et al*. 2009. On the other hand, there was a higher number of mice inoculated with high virulence isolates that showed specific anti-rNcSAG4 antibodies in the pregnant mouse model. Similarly, antibodies against rNcBSR4 and rNcSRS9 were only detected by some dams inoculated with high virulence isolates, such as Nc-Spain 4H, Nc-Spain 7 and Nc-Liverpool, although specific antibodies against these two proteins were not detected in the non-pregnant model. Because NcSAG4 is expressed early during the tachyzoite-bradyzoite conversion (Fernández-García *et al*. 2006), this protein could be exposed to the host immune system. On the contrary, the bradyzoite-specific late-expressed proteins NcBSR4 and NcSRS9 have only been detected in mature cysts within host tissues (Risco-Castillo *et al*. 2007, 2011). In this sense, NcBSR4 and NcSRS9 would be only exposed to the host immune system after a cyst ruptured to reactivate the infection. In accordance with this model, several experiments with TgSRS9 in transgenic parasites demonstrated that this protein plays a role in parasite persistence and posterior parasite reactivation (Kim and Boothroyd, 2005; Kim *et al*. 2007). Indeed, whereas both *N. caninum* proteins were detected by immunohistochemical techniques in tissue cysts of cattle congenitally infected with the Nc- Spain 7 isolate, these proteins have rarely been detected in *in vitro* assays in which mainly vacuoles with a mixture of tachyzoites and bradyzoites instead of mature cysts were obtained (Risco-Castillo *et al*. 2007, 2011). The detection of these 3 bradyzoite-specific proteins by a few mice from groups inoculated with high-virulence isolates for which morbidity and mortality were recorded could be due to a spontaneous rupture of the cysts (Rettigner *et al*. 2004). The serological differences observed between the infections with the low-to-moderate virulence isolates and the high-virulence isolates might be not only attributed to their intra-organic distribution during infection and the final parasite burden in brain, but also to their different capacities for the tachyzoite-bradyzoite switch (Collantes-Fernández *et al*. 2006; Pereira García-Melo *et al*. 2010). Therefore, further investigations would be needed to clarify the differences in conversion rates among this panel of *N. caninum* isolates.

This association of virulence with the specific immune response observed in *N. caninum* infections is in accordance with the results obtained in studies of the closely related parasite *T. gondii*. Regarding the humoral immune response, Pardini *et al*. (2012) have

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recently reported that a high virulence strain in mice (RH; type I) induced earlier and more specific antibody levels against TgSAG1 compared with type II and type III strains in infected pigs. Moreover, Sousa *et al*. (2008) reported that TgGRA6 could potentially be used to serotype different *T. gondii* strains in human samples because higher antibody levels against TgGRA6 were detected in sera from patients infected with the high virulence strains (type I). Interestingly, Hill *et al*. (2012) reported that a higher number of genes involved in the host immune and inflammatory responses were up-regulated in mice infected with a type I strain. In fact, several *T. gondii* proteins have been identified as virulence factors, particularly the rhoptry protein kinases and pseudokinases that modulate pro-inflammatory host cytokine IL-12 and interferon-gamma (IFN-γ) responses (Saeij *et al*. 2006).

In conclusion, NcGRA7 and NcSAG4 are proteins clearly correlated with virulence, and, to a lesser extent, NcBSR4 and NcSRS9 proteins. Further analyses should be performed in order to verify the usefulness of NcGRA7 and NcSAG4 as predictive markers for virulence in an experimental bovine model of neosporosis. However, the utility of these proteins as serological markers in naturally infected cattle might be difficult to analyse due to several variables that may have an influence on the serological response (e.g. circulating isolate in the herd, parasite burden and parasite stage). Moreover, antibodies against anti-bradyzoite-specific late expressed proteins seem also to be related to virulence. Further functionality and host cellular immune response studies should be conducted, and other proteins involved in the tachyzoite lytic cycle (i.e. proteins involved in adhesion and invasion processes like surface proteins -NcSAG1, NcSRS2- dense granules -NcGRA1, NcGRA2, NcGRA6- microneme – NcMIC1, NcMIC3- and rhoptry -NcROP2- proteins) as well as proteins more abundant in high-virulence isolates (Regidor-Cerrillo *et al*. 2012) should also be considered for their potential roles in virulence.

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REFERENCES

Aguado-Martínez, A., Álvarez-García, G., Fernández-García, A., Risco-Castillo, V., Arnaiz-Seco, I., Rebordosa-Trigueros, X., Navarro-Lozano, V. and Ortega-Mora, L. M. (2008). Usefulness of rNcGRA7- and rNcSAG4-based ELISA tests for distinguishing primo-infection, recrudescence, and chronic bovine neosporosis. *Veterinary Parasitology* **157**, 182–195. doi: 10.1016/j.vetpar.2008.08.002.

Aguado-Martínez, A., Ortega-Mora, L. M., Álvarez-García, G., Rodríguez-Marco, S., Risco-Castillo, V., Marugán-Hernández, V. and Fernández-García, A. (2009*a*). Stage-specific expression of Nc SAG4 as a marker of chronic *Neospora caninum* infection in a mouse model. *Parasitology* **136**, 757–764. doi: 10.1017/S0031182009006076.

Aguado-Martínez, A., Álvarez-García, G., Fernández-García, A., Risco-Castillo, V., Marugán-Hernández, V. and Ortega-Mora, L. M. (2009*b*). Failure of a vaccine using immunogenic recombinant proteins rNcSAG4 and rNcGRA7 against neosporosis in mice. *Vaccine* **27**, 7331–7338. doi: 10.1016/j.vaccine.2009.09.050.

Aguado-Martínez, A., Álvarez-García, G., Schares, G., Risco-Castillo, V., Fernández-García, A., Marugán-Hernández, V. and Ortega-Mora, L. M. (2010). Characterisation of NcGRA7 and NcSAG4 proteins: immunolocalisation and their role in the host cell invasion by *Neospora caninum* tachyzoites. *Acta Parasitologica* **55**, 304–312. doi: 10.2478/s11686-010-0056-9.

Al-Qassab, S., Reichel, M. P., Ivens, A. and Ellis, J. T. (2009). Genetic diversity amongst isolates of *Neospora caninum*, and the development of a multiplex assay for the detection of distinct strains. *Molecular and Cellular Probes* **23**, 132–139. doi: 10.1016/j.mcp.2009.01.006.

Álvarez-García, G., Pitarch, A., Zaballos, A., Fernández-García, A., Gil, C., Gómez-Bautista, M., Aguado-Martínez, A. and Ortega-Mora, L. M. (2007). The NcGRA7 gene encodes the immunodominant 17 kDa antigen of *Neospora caninum*. *Parasitology* **134**, 41–50. doi: 10.1017/S0031182006001284.

Bartley, P. M., Wright, S., Sales, J., Chianini, F., Buxton, D. and Innes, E. A. (2006). Long-term passage of tachyzoites in tissue culture can attenuate virulence of *Neospora caninum in vivo*. *Parasitology* **133**, 421–432. doi: 0.1017/S0031182006000539.

Basso, W., Schares, S., Barwald, A., Herrmann, D. C., Conraths, F. J., Pantchev, N., Vrhovec, M. G. and Schares, G. (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. *Veterinary Parasitology* **160**, 43–50. doi: 10.1016/j.vetpar.2008.10.085.

Caspe, S. G., Moore, D. P., Leunda, M. R., Cano, D. B., Lischinsky, L., Regidor-Cerrillo, J., Alvarez-Garcia, G., Echaide, I. G., Bacigalupe, D., Ortega Mora, L. M., Odeon, A. C. and Campero, C. M. (2012). The *Neospora caninum*-Spain 7 isolate induces placental damage, fetal death and abortion in cattle when inoculated in early gestation. *Veterinary Parasitology* **189**(2–4), 171–811. doi: 10.1016/j.vetpar.2012.04.034.

Collantes-Fernandez, E., Arrighi, R. B., Alvarez-Garcia, G., Weidner, J. M., Regidor-Cerrillo, J., Boothroyd, J. C., Ortega-Mora, L. M. and Barragan, A. (2012). Infected dendritic cells facilitate systemic dissemination and transplacental passage of the obligate intracellular parasite *Neospora caninum* in mice. *PloS One* **7**, e32123. doi: 10.1371/journal.pone.0032123.

Collantes-Fernández, E., López-Pérez, I., Álvarez-García, G. and Ortega-Mora, L. M. (2006). Temporal distribution and parasite load kinetics in blood and tissues during *Neospora caninum* infection in mice. *Infection and Immunity* **74**, 2491–2494. doi: 10.1128/IAI.74.4.2491-2494.2006.

Dubey, J. P. and Schares, G. (2011). Neosporosis in animals – the last five years. *Veterinary Parasitology* **180**, 90–108. doi: 10.1016/j.vetpar.2011.05.031.

Dubey, J. P., Buxton, D. and Wouda, W. (2006). Pathogenesis of bovine neosporosis. *Journal of Comparative Pathology* **134**, 267–289. doi: 10.1016/j.jcpa.2005.11.004.

Dubey, J. P., Schares, G. and Ortega-Mora, L. M. (2007). Epidemiology and control of neosporosis and *Neospora caninum*. *Clinical Microbiology Reviews* **20**, 323–367. doi: 10.1128/CMR.00031-06.

Fernández-García, A., Risco-Castillo, V., Zaballos, A., Álvarez-García, G. and Ortega-Mora, L. M. (2006). Identification and molecular cloning of the *Neospora caninum* SAG4 gene specifically expressed at bradyzoite stage. *Molecular and Biochemical Parasitology* **146**, 89–97. doi: 10.1016/j.molbiopara.2005.08.019.

Hemphill, A., Felleisen, R., Connolly, B., Gottstein, B., Hentrich, B. and Muller, N. (1997). Characterization of a cDNA-clone encoding Nc-p43, a major *Neospora caninum* tachyzoite surface protein. *Parasitology* **115**, 581–590. doi: 10.1017/S0031182097001650.

Hemphill, A., Gajendran, N., Sonda, S., Fuchs, N., Gottstein, B., Hentrich, B. and Jenkins, M. (1998). Identification and characterisation of a dense granule-associated protein in *Neospora caninum* tachyzoites. *International Journal for Parasitology* **28**, 429–438. doi: 10.1016/S0020-7519(97)00193-8.

Hill, R. D., Gouffon, J. S., Saxton, A. M. and Su, C. (2012). Differential gene expression in mice infected with distinct *Toxoplasma* strains. *Infection and Immunity* **80**, 968–974. doi: 10.1128/IAI.05421-11.

Jiménez-Ruiz, E., Álvarez-García, G., Aguado-Martínez, A., Salman, H., Irache, J. M., Marugán-Hernández, V. and Ortega-Mora, L. M. (2012). Low efficacy of NcGRA7, NcSAG4, NcBSR4 and NcSRS9 formulated in poly-epsilon-caprolactone against *Neospora caninum* infection in mice. *Vaccine* **30**, 4983–4992. doi: 10.1016/j.vaccine.2012.05.033.

Kim, S. K. and Boothroyd, J. C. (2005). Stage-specific expression of surface antigens by *Toxoplasma gondii* as a mechanism to facilitate parasite persistence. *Journal of Immunology* **174**, 8038–8048.

Kim, S. K., Karasov, A. and Boothroyd, J. C. (2007). Bradyzoite-specific surface antigen SRS9 plays a role in maintaining *Toxoplasma gondii* persistence in the brain and in host control of parasite replication in the intestine. *Infection and Immunity* **75**, 1626–1634. doi: 10.1128/IAI.01862-06.

Miller, C. M., Quinn, H. E., Windsor, P. A. and Ellis, J. T. (2002). Characterisation of the first Australian isolate of *Neospora caninum* from cattle. *Australian Veterinary Journal* **80**, 620–625. doi: 10.1111/j.1751-0813.2002.tb10967.x.

Pardini, L., Maksimov, P., Herrmann, D. C., Bacigalupe, D., Rambeaud, M., Machuca, M., More, G., Basso, W., Schares, G. and Venturini, M. C. (2012). Evaluation of an in-house TgSAG1 (P30) IgG ELISA for diagnosis of naturally acquired *Toxoplasma gondii* infection in pigs. *Veterinary Parasitology* **189**, 204–210. doi: 10.1016/j.vetpar.2012.4.014.

Pedraza-Díaz, S., Marugán-Hernández, V., Collantes-Fernández, E., Regidor-Cerrillo, J., Rojo-Montejo, S., Gómez-Bautista, M. and Ortega-Mora, L. M. (2009). Microsatellite markers for the molecular characterization of *Neospora caninum*: application to clinical samples. *Veterinary Parasitology* **166**, 38–46. doi: 10.1016/j.vetpar.2009.07.043.

Pereira García-Melo, D., Regidor-Cerrillo, J., Collantes-Fernández, E., Aguado-Martínez, A., Del Pozo, I., Minguijon, E., Gómez-Bautista, M., Aduriz, G. and Ortega-Mora, L. M. (2010). Pathogenic characterization in mice of *Neospora caninum* isolates obtained from asymptomatic calves. *Parasitology* **137**, 1057–1068. doi: 10.1017/S0031182009991855.

Pérez-Zaballos, F. J., Ortega-Mora, L. M., Álvarez-García, G., Collantes-Fernández, E., Navarro-Lozano, V., García-Villada, L. and Costas, E. (2005). Adaptation of *Neospora caninum* isolates to cell-culture changes: an argument in favor of its clonal population structure. *Journal of Parasitology* **91**, 507–510. doi: 10.1645/GE-381R1.

Quinn, H. E., Miller, C. M., Ryce, C., Windsor, P. A. and Ellis, J. T. (2002). Characterization of an outbred pregnant mouse model of *Neospora caninum* infection. *Journal of Parasitology* **88**, 691–696. doi: 10.1645/0022-3395(2002)088[0691:COAOPM]2.0.CO;2.

Rettigner, C., De Meerschman, F., Focant, C., Vanderplasschen, A. and Losson, B. (2004). The vertical transmission following the reactivation of a *Neospora caninum* chronic infection does not seem to be due to an alteration of the systemic immune response in pregnant CBA/Ca mice. *Parasitology* **128**, 149–160.

Regidor-Cerrillo, J., Pedraza-Díaz, S., Gómez-Bautista, M. and Ortega-Mora, L. M. (2006). Multilocus microsatellite analysis reveals extensive genetic diversity in *Neospora caninum*. *Journal of Parasitology* **92**, 517–524. doi: 10.1645/GE-713R.1.

Regidor-Cerrillo, J., Gómez-Bautista, M., Del Pozo, I., Jiménez-Ruiz, E., Aduriz, G. and Ortega-Mora, L. M. (2010). Influence of *Neospora caninum* intra-specific variability in the outcome of infection in a pregnant BALB/c mouse model. *Veterinary Research* **41**, 52. doi: 10.1051/vetres/2010024.

Regidor-Cerrillo, J., Gómez-Bautista, M., Sodupe, I., Aduriz, G., Álvarez-García, G., Del Pozo, I. and Ortega-Mora, L. M. (2011). *In vitro* invasion efficiency and intracellular proliferation rate comprise virulence-related phenotypic traits of *Neospora caninum*. *Veterinary Research* **42**, 41. doi: 10.1186/1297-9716-42-41.

Regidor-Cerrillo, J., Álvarez-García, G., Pastor-Fernández, I., Marugán-Hernández, V., Gómez-Bautista, M. and Ortega-Mora, L. M. (2012). Proteome expression changes among virulent and attenuated *Neospora caninum* isolates. *Journal of Proteomics* **75**, 2306–2318. doi: 10.1016/j.jprot.2012.01.039.

Risco-Castillo, V., Fernández-García, A., Zaballos, A., Aguado-Martínez, A., Hemphill, A., Rodríguez-Bertos, A., Álvarez-García, G. and Ortega-Mora, L. M. (2007). Molecular characterisation of BSR4, a novel bradyzoite-specific gene from *Neospora caninum*. *International Journal for Parasitology* **37**, 887–896. doi: 10.1016/j.ijpara.2007.02.003.

Risco-Castillo, V., Marugán-Hernández, V., Fernadez-García, A., Aguado-Martínez, A., Jiménez-Ruiz, E., Rodriguez-Marco, S., Álvarez-García, G. and Ortega-Mora, L. M. (2011). Identification of a gene cluster for cell-surface genes of the SRS superfamily in *Neospora caninum* and characterization of the novel SRS9 gene. *Parasitology* **138**, 1832–1842. doi: 10.1017/S0031182011001351.

Rojo-Montejo, S., Collantes-Fernández, E., Blanco-Murcia, J., Rodríguez-Bertos, A., Risco-Castillo, V. and Ortega-Mora, L. M. (2009). Experimental infection with a low virulence isolate of *Neospora caninum* at 70 days gestation in cattle did not result in foetopathy. *Veterinary Research* **40**, 49. doi: 10.1051/vetres/2009032.

Rojo-Montejo, S., Collantes-Fernandez, E., Rodriguez-Marcos, S., Perez-Zaballos, F., Lopez-Perez, I., Prenafeta, A. and Ortega-Mora, L. M. (2011). Comparative efficacy of immunization with inactivated whole tachyzoites versus a tachyzoite-bradyzoite mixture against neosporosis in mice. *Parasitology* **138**, 1372–1383. doi: 10.1017/S0031182011001156.

Saeij, J. P., Boyle, J. P., Coller, S., Taylor, S., Sibley, L. D., Brooke-Powell, E. T., Ajioka, J. W. and Boothroyd, J. C. (2006). Polymorphic secreted kinases are key virulence factors in toxoplasmosis. *Science* **314**, 1780–1783. doi: 10.1126/science.1133690.

Schock, A., Innes, E. A., Yamane, I., Latham, S. M. and Wastling, J. M. (2001). Genetic and biological diversity among isolates of *Neospora caninum*. *Parasitology* **123**, 13–23. doi: 10.1017/S003118200100796X.

Sousa, S., Ajzenberg, D., Vilanova, M., Costa, J. and Darde, M. L. (2008). Use of GRA6-derived synthetic polymorphic peptides in an immunoenzymatic assay to serotype *Toxoplasma gondii* in human serum samples collected from three continents. *Clinical and Vaccine Immunology* **15**, 1380–1386. doi: 10.1128/CVI.00186-08.

Walsh, C. P., Vemulapalli, R., Sriranganathan, N., Zajac, A. M., Jenkins, M. C. and Lindsay, D. S. (2001). Molecular comparison of the dense granule proteins GRA6 and GRA7 of *Neospora hughesi* and *Neospora caninum*. *International Journal for Parasitology* **31**, 253–258. doi: 10.1016/S0020-7519(00)00169-7.

Zhao, J. C., Zhao, Z. D., Wang, W. and Gao, X. M. (2005). Prokaryotic expression, refolding, and purification of fragment 450-650 of the spike protein of SARS-coronavirus. *Protein Expression and Purification* **39**, 169–174. doi: 10.1016/j.pep.2004.10.004.