Highlights

- Five *Neospora* potential diagnostic antigens were compared by iELISA.
- Higher levels of NcSAG1 and NcGRA7 antibodies were detected in aborting cows.
- Higher antibody levels against NcSAG1 and NcGRA7 at the delivery time were observed.
- No marked differences in antibody levels were noted in paralytic form of calves.
- A higher level of anti-NcSAG1 antibodies was associated with *Neospora* abortion.

Evaluation of *Neospora caninum* serodiagnostic antigens for bovine neosporosis

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ABSTRACT

Abortion and reproductive failure caused by *Neospora caninum* infection has a dramatic negative economic impact on the cattle industry. To date, no definitive serodiagnostic tool for assessing *N. caninum* abortion has been reported. In this study, we evaluated the diagnostic performance of numerous *N. caninum* antigens in relation to abortion in cattle. Five recombinant proteins with potential as diagnostic antigens (NcGRA6, NcGRA7, NcGRA14, NcCyP, and NcSAG1) were compared by indirect enzyme-linked immunosorbent assay (iELISA) using sera from mice and cattle experimentally infected with *N. caninum*. The best-performing three antigens (NcSAG1, NcGRA7, and NcGRA6) were evaluated by IgG-iELISAs to assess their utility in diagnosing *Neospora* abortion using sera from confirmed *N. caninum*-aborted dams based on immunohistochemical assays (IHC). Additionally, all samples were tested using a commercial *N. caninum* antibody competitive ELISA (cELISA). The iELISAs against both NcSAG1 and NcGRA7 could efficiently distinguish IHC positive and negative samples compared with iELISAs against NcGRA6 and the cELISA. Furthermore, antibody levels against NcSAG1 and NcGRA7 were significantly higher in aborting cows comparing with infected but non-aborted dams in a herd experiencing a *Neospora* abortion outbreak. Tracking the dynamics of antibody levels during pregnancy revealed a marked increase in NcSAG1- and NcGRA7-specific antibodies at the last trimester of pregnancy. In contrast, no marked differences in antibody levels against either antigen were noted in neurologically symptomatic calves compared with non-symptomatic infected calves. Our data suggests NcSAG1 and NcGRA7 as indicators for *Neospora* abortion.

Keywords

Neospora caninum; Diagnosis; Neosporosis; Abortion; Cattle; Antigen

1. Introduction

Neospora caninum is a protozoan parasite with a wide host range. Members of the family *Canidae* are the definitive hosts in which the parasite replicates sexually, while many species of domestic and wild animals can act as intermediate hosts. Abortion is the most significant sign of the disease in cattle. Other symptoms including neurological disorders, inability to rise, and below average birth weight of newborn calves. Unlike the definitive host, vertical transmission of the parasite from infected dams to their progeny is the main route of infection [1]. Congenitally infected calves can pass the infection onto their progeny and perpetuate the vertical transmission cycle of the parasite in herds [2]. By contrast, horizontal infection via ingestion of contaminated food or water containing fecal oocysts from infected dogs is primary route of epidemic transmission [3,4]. The annual economic burden of *Neospora* infection is estimated at US $1.1 million in New Zealand beef farms and US $546.3 million in US dairy cattle [5].

Despite extensive efforts to develop effective vaccines or pharmacological treatments for neosporosis [6], progress has been slow. Improved diagnostic methods represent an effective and accessible means to control neosporosis. Control measures based on diagnosis aim to minimize vertical transmission by selective breeding and limiting horizontal transmission through application of hygienic disposal procedures for aborted fetal and maternal tissue. Cases of *Neospora* abortion can be confirmed through detection of *N. caninum* tachyzoites in fetal or maternal lesions, while demonstration of specific antibodies in maternal sera or fetal fluids provides strong evidence that abortion might be associated with *N. caninum*. The immunohistochemistry assay (IHC) is a routine diagnostic test for detecting *N. caninum* antigens in infected tissue [7,8]. However, low sensitivity of IHC, especially in autolyzed tissue, has been reported [9].

Detection of *N. caninum* antibodies can be achieved through many serological tests including immunofluorescence antibody test (IFAT) and indirect enzyme-linked immunosorbent assay (iELISA). Although IFAT using whole fixed tachyzoites is the most reliable serological test for detection of *Neospora* antibodies, high cost and the need for specialized equipment and expertise have limited its use [10]. The iELISA against recombinant antigens is a common serological test for detection of *Neospora* infection in large-scale surveillance studies. In the last 5 years, many recombinant antigens with good diagnostic agreement and high performance have been identified [11].

N. caninum surface antigen 1 (NcSAG1) is an immunodominant antigen expressed in tachyzoites and downregulated during the tachyzoite-to-bradyzoite conversion [12]. Recombinant NcSAG1 based-iELISA is an effective serodiagnostic tool for detection of *N. caninum* infection in cattle and dogs [13, 14]. Dense granule protein 7 (NcGRA7) is one of the best-studied *N. caninum* antigens. NcGRA7 is expressed in both the tachyzoite and bradyzoite stages of *N. caninum* and exhibited good performance for detection of specific antibodies in infected animals [15, 16,17]. The role of NcGRA7 in regulation of *N. caninum* pathogenesis through modulation of host immune responses was recently clarified [18]. The diagnostic performance of dense granule protein 6 (NcGRA6) was reported for cattle sera [19]. The rNcGRA6 protein is an efficient immunomodulator and potential vaccine candidate for *N. caninum* infection in mouse models [20]. *N. caninum* cyclophilin antigen (NcCyP) is a secretory protein which triggers production of host interferon-gamma (IFN- γ), contributes to host cell migration [21,22], and induces potent protection against *N. caninum* infection in mice in a Toll-like receptor 2-dependent manner [23]. Dense granule protein 14 (NcGRA14) is a recently-described *N. caninum* protein and no reports have examined its antigenic performance [24].

A limited number of previous studies have been conducted to evaluate the diagnostic performance of recombinant antigen iELISAs for serological confirmation of *N. caninum* as a causative agent of abortion in cattle [25,15]. Accordingly, we conducted this study to compare the serodiagnostic performance of numerous *N. caninum* antigens (NcSAG1, NcGRA6, NcGRA7, NcGRA14 and cyclophilin), and to investigate the diagnostic utility of selected antigens for *Neospora*-induced abortion in cattle.

2. Materials and Methods

2.1. Ethics statement

All animal experiments strictly followed the recommendations of the Guide for the Care and Use of Laboratory Animals of the Ministry of Education, Culture, Sports, Science and Technology, Japan. The study protocol was approved by the Committee on the Ethics of Animal Experiments at the Obihiro University of Agriculture and Veterinary Medicine (permission numbers: 29-58, 8-15, 18-40, 119-3, 19-51). For mice, general anesthesia with isoflurane was applied prior to painful experimental procedures.

2.2. Parasites and host cell cultures

The *N. caninum* (Nc-1) strain was propagated in Vero cells (African green monkey kidney epithelial cells) cultured in Eagle's minimum essential medium (Sigma, St Louis, MO, USA) supplemented with 8% heat-inactivated fetal bovine serum (FBS; Nichirei Biosciences, Tokyo, Japan) and 1% streptomycin–penicillin (Sigma). For tachyzoite purification, sterile phosphate-buffered saline (PBS) was used to wash parasites in host cell debris, and then the infected cell monolayer was separated using a cell scraper (BD Bioscience, San Jose, CA, USA). The cell pellet was resuspended in RPMI 1640 medium (Sigma) using a 27-gauge needle and passed through a 5 μm filter (Millipore, Bedford, MA, USA).

2.3. Preparation of recombinant antigens

This study used five *N. caninum* recombinant antigens: NcSAG1, NcGRA7, NcGRA6, NcGRA14, and NcCyP. Specific primers containing suitable restriction enzyme sites were designed to amplify target genes, and the target proteins were expressed as described previously (Table S1) with slight modifications. In brief, PCRs were performed using *N. caninum* cDNA (Nc-1 strain) as a template. The digested PCR products were purified from

agarose gels and cloned into pGEX-4T-1 or pGEX-4T3 expression vectors treated with the same restriction enzymes. Successful insertion was confirmed by sequencing. All recombinant proteins were expressed in *Escherichia coli* BL21 (DE3) cells as glutathione S-transferase (GST) fusions (New England BioLabs Inc., Ipswich, MA, USA). Expression was induced using 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) (Wako Inc., Osaka, Japan) for 6 h at 37°C (NcSAG1, NcGRA7, and NcCyP), using 0.1 mM IPTG at 37°C (NcGRA6), or using 0.1 mM IPTG at 27°C (NcGRA14). Bacterial cells were harvested and the pellets were suspended in sonication buffer (50 mM Tris-HCl, pH 8, 50 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA) and 1 mM dithiothreitol) then centrifuged at 7,180×*g* at 4°C for 10–15 min. Lysozyme (final concentration of 500 μg/mL) and Triton X-100 (10%) in PBS were added followed by incubation on ice for 1 h. The lysate was applied to Glutathione Sepharose 4B beads (GE Healthcare Life Sciences, Buckinghamshire, England) according to the manufacturer's instructions. Briefly, the supernatant was incubated with washed beads overnight at 4°C (NcSAG1, NcGRA7, and NcCyP) or for 30 min at room temperature (NcGRA14 and NcCyP) with gentle rotation. GST fusion proteins were eluted with elution buffer (100 mM Tris-HCl, pH 8.0, containing 100 mM NaCl, 5 mM EDTA, and 25 mM reduced glutathione powder; Wako Inc). In the case of NcCyP, the GST tag was removed with thrombin protease (GE Healthcare) according to the manufacturer's instructions. The quantity and purity of each protein were determined by SDS-PAGE followed by staining with Coomassie Brilliant Blue R250 (MP Biomedicals Inc., Illkirch-Graffenstaden, France). The protein concentrations were assayed with a bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Inc., Rockford, IL, USA). The recombinant proteins (NcSAG1-GST, NcGRA6-GST, NcGRA7-GST, NcGRA14-GST, NcCyP and GST) had apparent molecular weights (55, 43.7, 54, 50, 20.5, and 26 kDa, respectively), consistent with the expected molecular weights (S1 Fig).

2.4. Serum samples

2.4.1. Sera from experimentally-infected animals

Antigens (NcGRA6, NcGRA7, NcGRA14, NcCyP, and NcSAG1) were evaluated using sera from experimentally-infected mouse and cattle. For the preparation of mouse sera, female BALB/c mice (8 weeks old, n = 4 for each group) were purchased from Clea Japan. Mice were intraperitoneally inoculated with *N. caninum* or *T. gondii* tachyzoites (1×10^5 and 1×10^3 tachyzoites, respectively) 1 week later. Serum samples were harvested from mouse blood samples collected at 0 and 7 weeks post-infection. Four male Holstein calves at 2–4 months of age were inoculated intravenously with 1×10^7 *N. caninum* (Nc-1 strain) tachyzoites. Blood was collected 13 days prior to and 28 days after infection and used as a negative and positive control, respectively. The reactivity of all sera was confirmed using commercial IFAT slides (VMRD, Pullman, WA, USA) and iELISA against NcSAG1 [26].

2.4.2. Field serum samples

The antigenic properties of highly diagnostic antigens (NcSAG1, NcGRA7 and NcGRA6) were validated by iELISA using field cattle sera (n = 164) collected from aborted dams in different herds in the Tokachi subprefecture of Japan from 2010 to 2018. Fetal tissues from aborted dams were collected by Tokachi Livestock Hygiene Service Center and tested for *N. caninum* antigens using IHC. In addition, their mother's sera were tested for seropositivity to *N. caninum* by IFAT (VMRD, Pullman, WA, USA).

Outbreak of an abortion epidemic on a dairy cattle farm located in Shihoro town, Hokkaido, Japan was monitored by sampling sera from 277 cattle of different physiological status (2010). About 171 cows were lactating, 74 were at the dry stage, and 32 animals were

pregnant for the first time. A total of 57 dams of all stages aborted, while the remainder did not abort. The samples were collected within one month of the beginning of the abortion outbreak.

To track the dynamic levels of NcSAG1 and NcGRA7-specific antibodies during pregnancy, blood samples were collected from pregnant dams (n = 36) on the same farm in Shihoro every100 days for 400 days (2012 to 2013). The first sampling was conducted simultaneously for all cattle in spite of different pregnancy date of each case, which ranged from day 14 before pregnancy to day 135 of pregnancy. Collected sera from first sampling were tested by rNcSAG1- and rNcGRA7-based iELISAs for detection of specific antibodies against *N. caninum*. According to the results, dams were grouped into *N. caninum*-seropositive and seronegative animals. Additionally, the dams were divided into three groups according to their history of abortion (see Fig. 4): (i) Group A, negative to *Neospora* infection and abortion; (ii) group B, with *N. caninum* infection confirmed at first sampling and no history of abortion; and (iii) group C, infected with *N. caninum* with a history of abortion. Notably, all aborted dams (n = 5) were positive for *N. caninum*. We did not detect any animals with a history of abortion that were seronegative against *N. caninum* (data not shown).

Additionally, sera of neurologically symptomatic (n = 41) and non-symptomatic (n = 16) calves younger than 6 months of age were obtained from animal hospitals at the Obihiro University of Agriculture and Veterinary Medicine, Japan (2006 to 2011). The sera were used to evaluate levels of specific anti-NcSAG1 and anti-NcGRA7 antibodies and their association with neurological disorders.

2.5. iELISA

iELISAs were performed as reported previously [26] with slight modifications. In brief, a uniform amount of all recombinant proteins and GST (50 µL of 0.1 µM) was added to each well of a 96-well microtiter plate (Nunc, Denmark). Antigens were prepared in coating buffer

(50 mM carbonate-bicarbonate buffer, pH 9.6) and incubated overnight at 4°C in the plate. The following day, the plates were washed once with PBST (0.05% Tween-20 in PBS) and blocked with 100 µL of 3% skim milk dissolved in PBS (PBS-SM) for 1 h at 37 °C. After another wash, 50 µL of the test sera (experimental mouse sera, 1:600; experimental cattle sera, 1:300; field cattle sera, 1:300) were diluted with PBS-SM and added to duplicate wells. The plates were incubated again for 1 h at 37°C. After six washes with PBST, 50 µL of horseradish peroxidase-conjugated goat anti-mouse IgG1 or IgG2a or rabbit anti-bovine IgG1, IgG2 or IgG antibodies were added to plates (Bethyl Laboratories, Montgomery, TX, USA). The secondary antibodies were diluted 1:15,000 in PBS-SM for mouse sera, 1:10,000 for experimentally-infected cattle sera or 1:5,000 for field cattle sera and incubated in the plates at 37°C for 1 h. The plates were washed again six times with PBST before addition of substrate [0.1 M citric acid, 0.2 M sodium phosphate, 0.003% H_2O_2, and 0.3 mg/mL 2, 2-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid); Sigma–Aldrich, St. Louis, MO, USA] to each well. After incubating at room temperature for 1 h, absorbance at 415 nm was measured using a microplate reader (MTP-120; Corona, Tokyo, Japan). Absorbance values for rNcSAG1, rNcGRA6, rNcGRA7, and rNcGRA14 antigens were determined after subtraction of the optical density for GST at 415 nm (OD415). Cutoff values for iELISA were estimated using negative control *N. caninum* cattle sera (n = 9) kept in our laboratory. To overcome plate to plate variation, we distributed the negative control samples among all used plates simultaneously for the calculation of a representative cutoff value. The standard positive and negative sera were confirmed with a commercial IFAT (VMRD Inc., Pullman, WA, USA). The commercial *N. caninum* antibody competitive ELISA (cELISA) antibody test kit was purchased from VMRD Inc.

2.6. Statistical analysis

225 Unless described, data were analyzed using GraphPad Prism 5 software (GraphPad
226 Software Inc., La Jolla, CA, USA). For statistical analysis, one-way analysis of variance
227 (ANOVA) followed by the Tukey–Kramer test or unpaired two-tailed *t* test was performed. A
228 *P* value < 0.05 was considered statistically significant. Degrees of statistical significance are
229 shown as different letters or as asterisks (*) defined in each figure legend.
230
231

3. Results

3.1. Assessment of recombinant antigens using sera from experimentally-infected animals

First, the diagnostic performance of antigens such as NcGRA6, NcGRA7, NcGRA14, NcCyP and NcSAG1 was evaluated using control mouse and cattle sera in iELISAs against each recombinant antigen. The highest reactivity was observed against NcSAG1 followed by NcGRA7 and NcGRA6 in *N. caninum*-infected mouse sera (IgG1 and IgG2a). No cross-reactivity was observed for sera of *T. gondii*-infected animals (Fig. 1A). Using sera from experimentally-infected cattle, the three antigens mentioned above were recognized by IgG1 antibody while binding by IgG2 was only observed for NcSAG1 (Fig. 1B). This result reflect the ability of NcSAG1 to induce both humoral and cellular immunity in cattle. No reactivity of mice or cattle sera was observed against NcGRA14 and NcCyP (Fig. 1). These results indicated the high efficacy of NcSAG1, NcGRA7 and NcGRA6 recombinant antigens for detection of *N. caninum* infection in mice and cattle.

3.2. Antigen validation using sera from aborted cattle

Aborted cattle sera (n=164) were collected from different farms in Tokachi subprefecture, Japan. Neosporosis was confirmed in aborted fetal tissue samples (n = 9) by IHC. We used the maternal sera to validate the performance of three highly diagnostic *Neospora* antigens (NcSAG1, NcGRA7 and NcGRA6) using iELISA, and compared these results against cELISA as a reference test. The three aforementioned antigens were differentially recognized in IgG-based iELISAs (S2 Fig.). From a total of 164 sera, 46 sera (28.1%) were positive against NcSAG1, 14 sera (8.5%) were positive against NcGRA7, and 12 sera (7.3%) were positive against NcGRA6. Specific *N. caninum* antibodies were detected in 22 samples (13.4%) using cELISA (S2 Table). Comparing the results of recombinant antigen-based iELISAs with the results of IHC (n = 9), NcSAG1 and NcGRA7-based iELISAs showed high agreement with

Neospora abortion-confirmed samples [9/9 (100%) and 8/9 (88.9%), respectively], while NcGRA6-based iELISA agreed for only 5/9 (55.6%) samples (S2 Table). Moreover, antibody levels against NcSAG1 and NcGRA7 antigens were significantly higher in the IHC- and iELISA-positive samples compared with samples positive by iELISA alone (Fig. 2). This result suggested that high levels of anti-NcSAG1 and anti-NcGRA7 antibodies were associated with bovine neosporosis. By contrast, cELISA was only able to detect 77.8% of neosporosis cases confirmed based on IHC (S2 Table).

3.3. Estimation of Neospora abortion prevalence

On a farm with an ongoing abortion outbreak, samples were collected from cattle of different physiological status (n = 277). Estimation of antibody levels against NcSAG1 and NcGRA7 revealed significantly higher levels of specific antibodies against both antigens in the sera of aborted cows comparing with non-aborted animals, in particular during the lactation period and in primiparous cows (Fig. 3). These results indicated the usefulness of both antigens as serological tools for estimation of *Neospora* abortion in cattle during abortion outbreaks.

3. 4. Dynamics of antibody levels in pregnant cattle

In order to investigate the potential role of the tested antigens in *Neospora*-induced abortion, antibody levels against NcSAG1 and NcGRA7 were tracked using serum samples collected from three groups of pregnant cattle with different serostatus to *N. caninum* infection and different histories of abortion (Fig. 4). All tested groups showed elevated antibody levels against NcSAG1 and NcGRA7 at the last trimester of pregnancy (188 to 283 days of pregnancy). In group A (n = 12), five animals (41.7%) showed marked changes in antibody levels against NcSAG1 (2.1–12.2-fold) compared with the first sampling, while three (25%) animals showed similar degrees of change in antibodies against NcGRA7 (2.2–8.3-fold).

Changes in antibody levels were apparent in group B (n = 19) in eight animals (42.1%) against NcSAG1 and in five animals (26.3%) against NcGRA7. The highest changes in antibody levels were observed in group C (n = 5): three animals (60%) had fold changes in antibody levels up to 33.0-fold against NcSAG1 and 11.2-fold against NcGRA7. The highest number of animals with marked increases in antibody levels occurred for NcSAG1, particularly in groups B and C in which *N. caninum* seropositivity were confirmed at first sampling. Higher levels of specific *N. caninum* antibodies in *Neospora* seropositive groups (B and C) indicated reactivation of *N. caninum* at this time (last trimester of pregnancy). These results suggested NcSAG1 as a marker for *Neospora* reactivation and subsequent prediction of *Neospora* abortion.

3.5. Antibody levels in calves with neurological symptoms

Further investigations of anti-NcSAG1 and anti-NcGRA7 antibody profiles in relation to another common clinical form of neosporosis were conducted. Serum samples were collected from neurologically symptomatic and asymptomatic calves. Fifty seven samples were tested by iELISA to measure IgG levels. High seropositive rate in neurologically symptomatic calves was detected against both antigens in comparison to non-neurologically cases, with no apparent differences in levels of anti-NcSAG1 and anti-NcGRA7 antibodies between the two groups (Fig. 5). Our results indicated that high levels of anti-NcSAG1 and anti-NcGRA7 antibodies were specifically associated with *Neospora* abortion rather than neurological symptoms.

4. Discussion

Estimation of *Neospora* abortion rates in cattle is required for the application of proper hygienic interventions against neosporosis. High seroprevalence rates estimated at 100% in some herds of dairy cattle have been reported [8]. Accordingly, specific antibodies can be detected in the sera of aborted dams and their fetuses even when *N. caninum* was not the cause of abortion. Definitive diagnosis of neosporosis requires a comprehensive diagnostic approach using immunohistochemical analysis of *N. caninum* antigens [8]. However, IHC is an invasive and postmortem test with limited sensitivity [9], and often shows marked cross-reactivity against *T. gondii* [27]. Additionally, the test is laborious and expensive. ELISA against recombinant antigens for detection of specific antibodies is a simple and rapid test requiring little serum and can be applied to live animals. Identification of *N. caninum* antigens as markers for *Neospora* abortion could overcome the demerits of IHC assays.

Serological estimation of *Neospora* abortion is an achievable goal if effective antigens are identified and specifically associated with the condition of abortion. Several ELISAs against recombinant antigens have been described to examine bovine sera for *N. caninum*-specific antibodies [11]. However, identifying appropriate cut-offs is essential for proper design of serological assays. *Neospora*-infected animals usually show lower levels of antibodies compared with aborted cases [28]. Thus, for identification of infected cattle, serological tests with higher sensitivity and lower cut-offs are required [4,29,30]. Recombinant antigen-based ELISAs against NcSAG1 and NcGRA7 are potential serological tools for detection of *N. caninum* infection in cattle [13,16], and dogs [14,17]. In the current study, NcSAG1 and NcGRA7 exhibited high performance and showed superiority compared with other antigens (NcGRA6, NcGRA14, and NcCyP) for detection of *Neospora* infection using sera from experimentally-infected mice and cattle. Consistently, marked changes in antibody levels against NcGRA6 suggested the utility of this antigen as a diagnostic marker for *N.*

caninum infection. By contrast, no reactivity of sera was observed against NcGRA14 and NcCyP. Thus, the NcSAG1, NcGRA7 and NcGRA6 antigens were selected for subsequent investigations using sera from *Neospora*-aborted cows.

The three antigens (NcSAG1, NcGRA7, and NcGRA6) were validated using aborted cattle sera by iELISA. The results were compared against a commercial ELISA kit as a reference test. The highest prevalence was observed for antibodies against NcSAG1, followed by NcGRA7 and NcGRA6. Moreover, both of NcSAG1 and NcGRA7 antigens showed significantly higher antibody levels in IHC-positive samples compared with IHC-negative samples. These results suggested that rNcSAG1- and rNcGRA7-based iELISAs were useful diagnostic tools for estimation of neosporosis. By contrast, cELISA detected *Neospora*-specific antibodies in IHC-positive samples less often compared with NcSAG1- and NcGRA7-based iELISAs, indicating the inappropriateness of the commercially used antigen for abortion cases. The VMRD test is a commercial *N. caninum* competitive ELISA test based on the GP65 surface antigen of tachyzoites. This assay is used extensively for detection of anti-*N. caninum* antibodies in the sera of domestic and wild animals. However, low specificity and agreement in addition to cross-reactivity with *Sarcocystis* spp. have been reported [31].

A more realistic investigation was conducted through testing the candidate antigens (NcSAG1 and NcGRA7) using cattle sera collected from a dairy herd experiencing an epidemic abortion outbreak. In fact, a dam may be seropositive for antibodies against *N. caninum*, even when abortion may have had another cause. Accordingly, a positive result of serological tests provides evidence of *N. caninum* infection, but not definitive proof that neosporosis caused abortion. However, animals that abort due to neosporosis often have higher *N. caninum*-specific antibody levels than infected but non-aborting dams [28,32,33]. Thus, definitive serodiagnosis can be accomplished by detecting statistically higher antibody levels in aborting cows compared with infected but non-aborting ones in herd with abortion outbreak. In the

current study, both NcSAG1 and NcGRA7 could differentiate statistically between aborted and non-aborted dams within a population of dams at risk. This finding demonstrates the usefulness of NcSAG1- and NcGRA7-based iELISA as serological tools to support the final judgment of *Neospora* abortion, while IHC still has a role in detecting parasite antigens in tissue samples, particularly in sporadic cases. Our previous study suggested the possibility of using serological testing for diagnosis of neosporosis as a cause of abortion among cattle [25]. We reported the utility of NcGRA7 as a candidate for detection of *N. caninum*-induced abortion in cattle. The related study also showed some evidence of the role of NcSAG1 in this process [25]. However, no significant differences have been recorded between aborting and non-aborting animals in terms of their antibody levels against NcSAG1. This slight variation regarding NcSAG1 reactivity might be attributable to differences in the timing of sample collection and the serological status of animals. The current study provides more comprehensive data and evidence and is based on a larger number of samples from animals with different physiological conditions.

Bradyzoite-to-tachyzoite reconversion of the *N. caninum* parasite usually takes place during pregnancy as a result of impaired immune systems of dams [34]. Our study tracked changes in antibody levels against anti-NcSAG1 and anti-NcGRA7 during pregnancy to define a suitable strategy for serodiagnosis based on these antigens. Periodic examination of maternal sera during pregnancy has shown an increase in levels of specific antibodies against both antigens at the last trimester of pregnancy. However, high antibody levels against NcGRA7 were observed only in sporadic cases, while many of the tested animals in the different groups (Fig. 4A, B and C) showed marked and sudden formation of specific antibodies against NcSAG1, particularly in *Neospora*-seropositive animals (Fig. 4B and C). The detection of antibody level against both antigens in the seronegative group during pregnancy (Fig. 4A) may indicate recent infection or reactivation of chronic infection associated with specific antibody

response below the detection limits at the first sampling. This result suggests that NcSAG1 could represent a new marker for *Neospora* reactivation and subsequently high antibody levels against NcSAG1 at the last trimester of pregnancy can be used for prediction of *Neospora* abortion. Accordingly, preventive measures are needed to deal with infected cases [35].

Up to 95% of live-born calves from *Neospora*-seropositive dams can be congenitally infected and clinically normal [36]. However, clinical signs, including neurologic signs have been reported in calves less than 4 months of age [37]. Thus, we investigated the specific reactivity against NcSAG1 and NcGRA7 of serum samples from neurologically and non-neurologically symptomatic calves with *N. caninum*. Our results showed no significant differences in the levels of *N. caninum*-specific antibody production between the two groups, indicating that antibodies against NcSAG1 and NcGRA7 were associated with *Neospora* abortion rather than neurological symptoms. Our results are consistent with those of Hiasa et al. (2012) who did not notice any marked differences in antibody levels against either antigen between asymptomatic and neurologically-symptomatic experimentally-infected mice. However, the same study recorded significantly higher levels of specific NcGRA7 antibodies in neurologically symptomatic dogs compared with non-neurologically symptomatic animals. This variation in antigen reactivity between mice, cattle and dogs may be attributable to species-specific differences.

5. Conclusions

Recently, significant advances have been made in serodiagnosis of *N. caninum* via specific antibody detection against antigens. In the current study, we developed antemortem serodiagnostic systems for the diagnosis of *Neospora*-induced abortion in cattle. On a herd level, demonstration of *Neospora* abortion can be achieved through the detection of significantly higher levels of specific anti-NcSAG1 and anti-NcGRA7 antibodies in aborted dams comparing to non-aborted cows in abortion outbreak as a result of horizontal infection, while the periodic examination of anti-NcSAG1 antibodies during pregnancy can identify *Neospora*-reactivation in sporadic aborted cases. Accordingly, our study identified NcSAG1 and NcGRA7-based iELISAs as serodiagnostic tools for detection and prediction of *N. caninum*-related abortion, and NcGRA6 as a possible candidate for serodiagnosis in field animals. Interestingly, neither NcSAG1 nor NcGRA7 antibody titers could discriminate between neurologically and non-neurologically symptomatic calves, reflecting the specific relevance of antibody titers against these targets for abortion. Higher antibody levels in infected or aborted animals and antibody dynamics associated with stage of pregnancy suggest the usefulness of NcSAG1 for further investigations as a marker of *Neospora* abortion.

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Conflict of interest

The authors declare that they have no financial or competing interests concerning this study.

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Fig. 1. Evaluation of *N. caninum*-derived recombinant antigens using sera from experimentally-infected animals. Antibody levels of IgG1 and IgG2a in sera of experimentally-infected mice (A) and antibody levels of IgG1 and IgG2 in sera of experimentally-infected cattle (B) were assessed against different *N. caninum* antigens (NcGRA6, NcGRA7, NcGRA14, NcCyP, and NcSAG1). Each bar represents the mean of the tested sera (nc, sera from uninfected mice: n = 4, *N. caninum*-infected mice: n = 4, *T. gondii*-infected mice: n = 4). The data are representative of two independent experiments with similar results. The different letters above the bars in the graphs indicate statistically significant differences among groups (one-way ANOVA with Tukey–Kramer *post hoc* analysis, $P < 0.05$).

Fig. 2: Determination of antibody levels of immunohistochemical assay (IHC)-positive samples and iELISA-positive samples using sera of aborted cattle. Comparison of antibody levels of IHC positive (+) and negative (–) in *Neospora*-positive samples determined using each iELISA. A: NcSAG1, B: NcGRA7, C: NcGRA6. Solid lines indicate average values. The significance of differences was analyzed using the Mann-Whitney *U* test because the data were non-normally distributed (*: $P < 0.05$, **: $P < 0.01$, ****: $P < 0.0001$).

Fig. 3: iELISA against recombinant antigens using sera from farms experiencing abortion outbreaks. iELISA data for a total of 277 sera from aborted and non-aborted cattle with different physiological status during abortion outbreaks are shown. Statistically significant differences were observed between the two groups in antibody levels against rNcSAG1 (A) and rNcGRA7 (B). Normally-distributed variables (Dry and First pregnancy) were compared using the Student's *t* test and non-normally distributed variables (Lactation and Total) were compared using the Mann-Whitney *U* test (*: $P < 0.05$, **: $P < 0.01$, ****: $P < 0.0001$). Solid red lines indicate the average values of samples. Dotted blue lines indicate the cutoff value.

564

565 **Fig. 4:** Dynamics of anti-NcSAG1 and anti- NcGRA7 antibody levels in pregnant cattle. Data
566 from 36 sera collected from pregnant dams with different serological status to *N. caninum* and
567 different histories of abortion are shown. Lines with different colors indicate different animals.
568 Red lines indicate cattle that experienced abortion or stillbirth during the sampling period.
569 Animals were classified into Groups A, B and C according to the table. ([a]): Abortion was
570 confirmed before the time of first sampling, ([b]): *Neospora*-specific antibodies were detected at
571 the first sampling by iELISA using either NcSAG1 or NcGRA7. The fold change was
572 calculated by dividing the OD value of each sampling point against initial sampling point for
573 the relevant animal tested by NcSAG1 or NcGRA7-based ELISA.

574

575 **Fig. 5:** Levels of anti-NcSAG1 (A) and anti-NcGRA7 (B) antibodies in neurologically
576 symptomatic calves. Results of iELISAs using sera from neurologically symptomatic (n = 41)
577 and non-symptomatic calves (n = 20) are shown. No significant differences were observed
578 between the two groups. Non-normally distributed variables (A) were compared using the
579 Mann-Whitney *U* test and normally-distributed variables (B) were compared using the
580 Student's *t* test. Solid red lines indicate the average values of samples. Dotted blue lines indicate
581 the cutoff value.

Supplemental information

S1 Table. Primers used for amplification of *N. caninum* antigens.

Antigen	Primers	Primer sequence	Restriction sites	Expression vector	Reference
NcSAG1	1-forward	5′-AC GAA TTC ATC AGA AAA ATC ACC T-3′	*Eco*RI	pGEX-4T-3	Chahan et. al., 2003 [1]
	2-reverse	5′-AC GAA TTC GAC CAA CAT TTT CAG C-3′	*Eco*RI		
NcGRA7	1-forward	5′-AC GAA TTC CGC TGG AGA CTT GGC A-3′	*Eco*RI	pGEX-4T-3	Abdelbaky et. al 2018 [2]
	2-reverse	5′-GT GAA TTC CTA TTC GGT GTC TAC TTC CTG-3′	*Eco*RI		
NcGRA6	1-forward	5′-AT GAA TTC ATG GATCCG GTT GAA TCC GTG GAG-3′	*Eco*RI	pGEX-4T-1	Fereig et. al., 2019 [3]
	2-reverse	5′-AT CTC GAG CTA TCT GTG ACG TGC CTG CTG CCG-3′	*Xho*I		
NcGRA14	1-forward	5′-GC GAA TTC ATG GGC TTG GGC GAG ATT TCG TAC-3′	*Eco*RI	pGEX-4T-1	Nishikawa et. al., 2018 [4]
	2-reverse	5′-AT CTC GAG CTA CCG AGA CTT GCC TCC GGA TGT-3′	*Xho*I		
NcCyP	1-forward	5’ -TA GGA TCC ATG GAA AAC GCC GGA GTC CAG-3’	*Bam*HI	pGEX-4T-1	Kameyama et. al., 2012 [5]
	2-reverse	5’-GC GAA TTC TTA CAA CAA ACC AAT GTC CGT-3’	*Eco*RI		

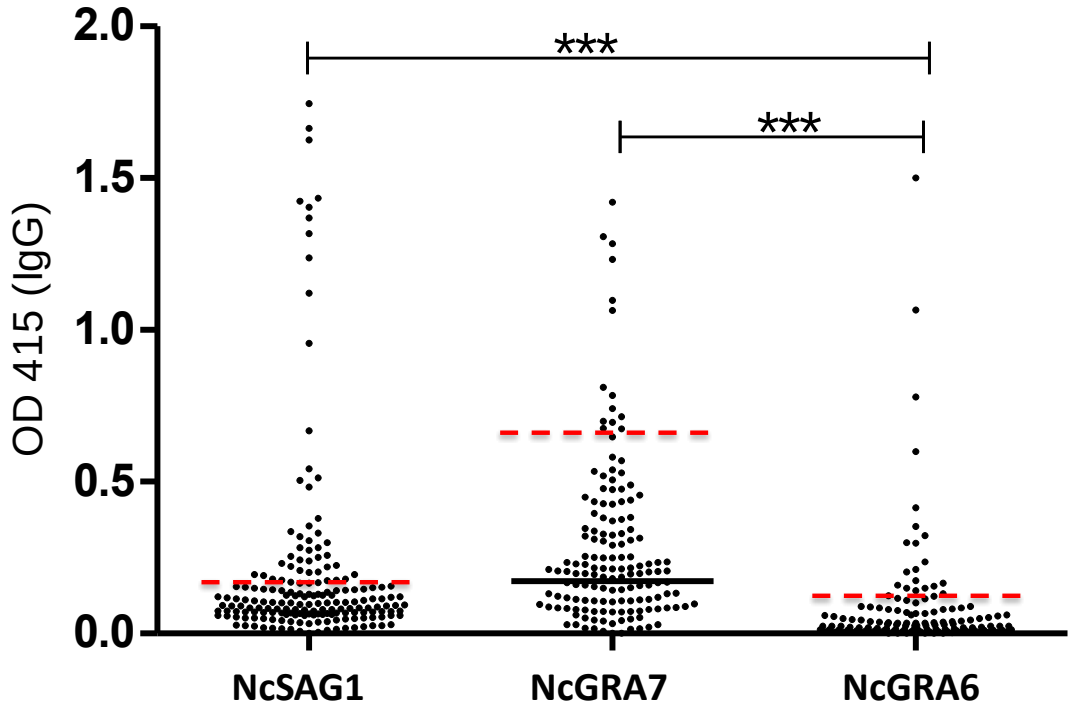
S2 Table. *N. caninum* seropositivity rates of aborting cattle using cELISA and iELISA against rNcSAG1, rNcGRA7 and rNcGRA6.

ELISA method	IHC-positive samples (n = 9)[a]	Total aborting cattle samples (n = 164)
cELISA	7/9 (77.8%)	22/164 (13.4%)
NcSAG1-iELISA	9/9 (100%)	46/164 (28.1%)
NcGRA7-iELISA	8/9 (88.9%)	14/164 (8.5%)
NcGRA6-iELISA	5/9 (55.6%)	12/164 (7.3%)

[a] Fetal tissues from the aborted dams were tested for *N. caninum* antigens using an immunohistochemical (IHC) assay used for diagnosis of neosporosis.

Supplemental figures

S1 Fig. SDS-PAGE of purified recombinant antigens. All recombinant proteins were GST fusions, except for NcCyP in which the GST tag was removed by thrombin protease digestion. KDa; kilodalton, LMW; low molecular weight marker.



S2 Fig. Validation of recombinant antigen-based iELISA using sera from aborting cattle. Comparison of *N. caninum* antigens (NcSAG1, NcGRA7 and NcGRA6) for analysis of aborted cattle sera (n = 164) using IgG-based iELISA. Higher prevalence rates were recorded against NcSAG1 (28.0%) followed by NcGRA7 (8.5%) and NcGRA6 (7.3%). Solid black lines indicate average values, while dotted red lines represent cut-off values which was calculated using negative control cattle sera (n = 9). An asterisk (***) indicates statistical differences by one-way ANOVA with Tukey–Kramer *post hoc* analysis ($P < 0.0001$).

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Figure 1

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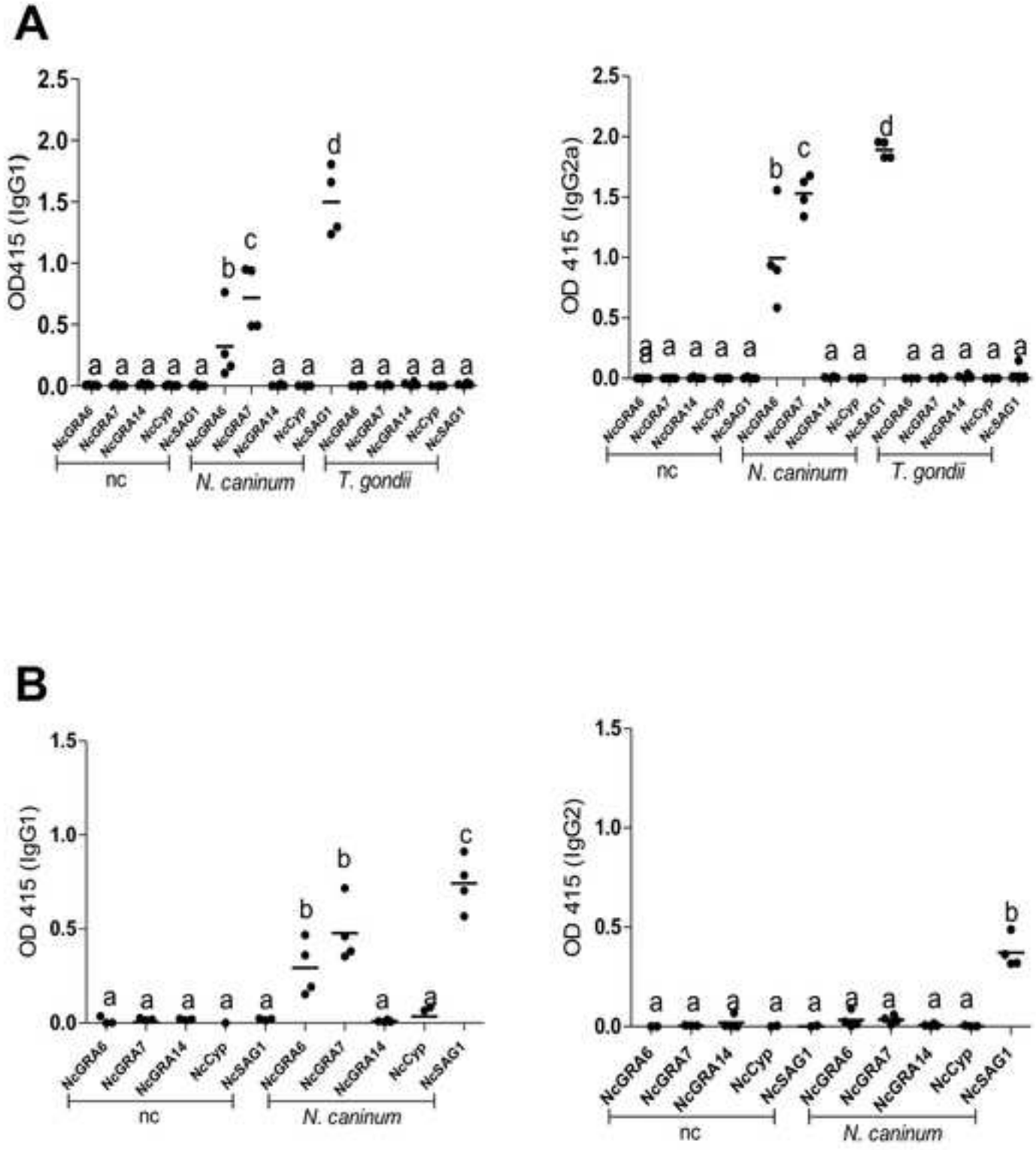
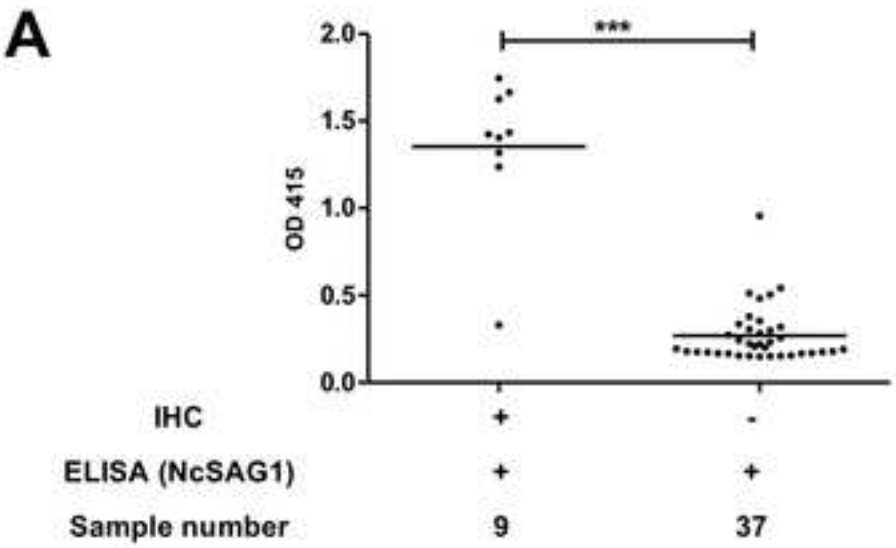
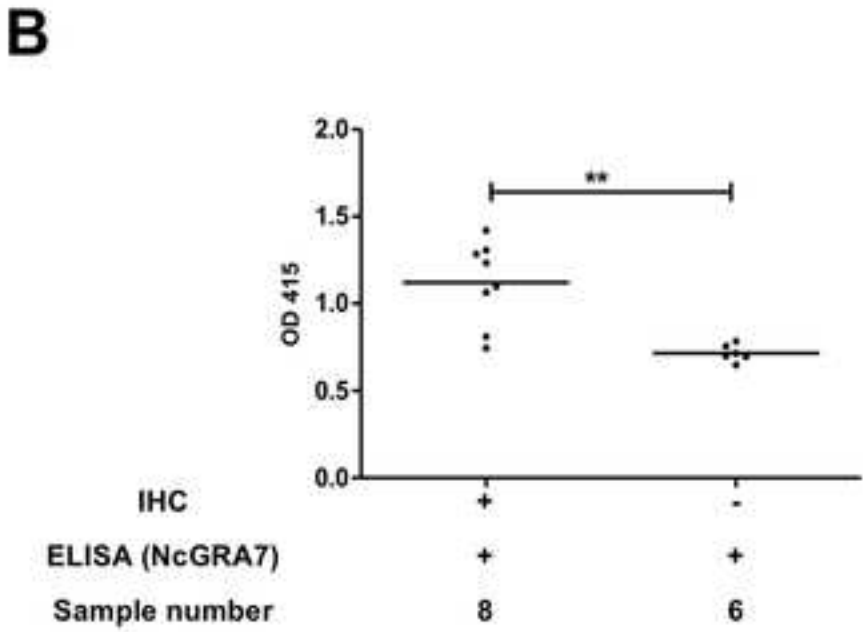


Figure 2





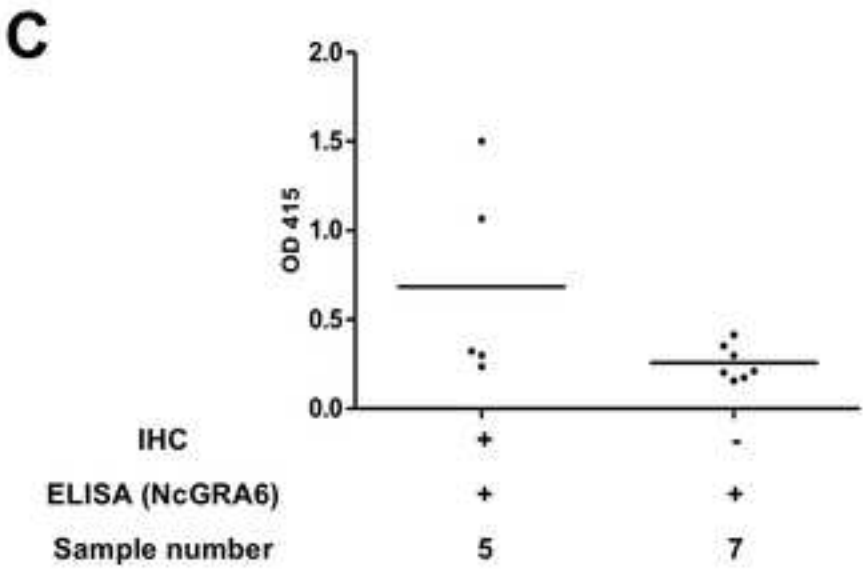
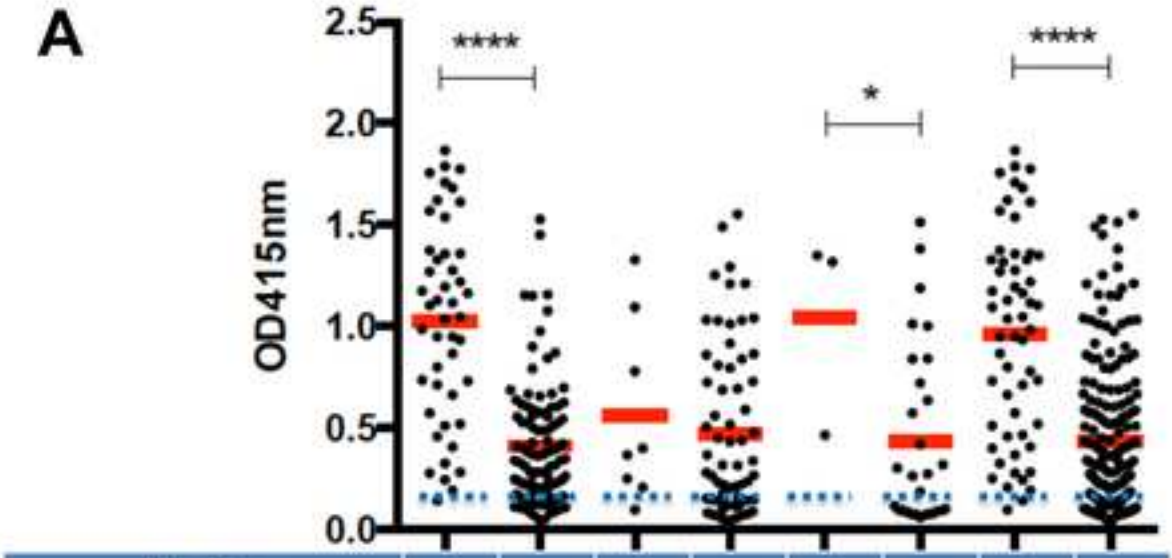
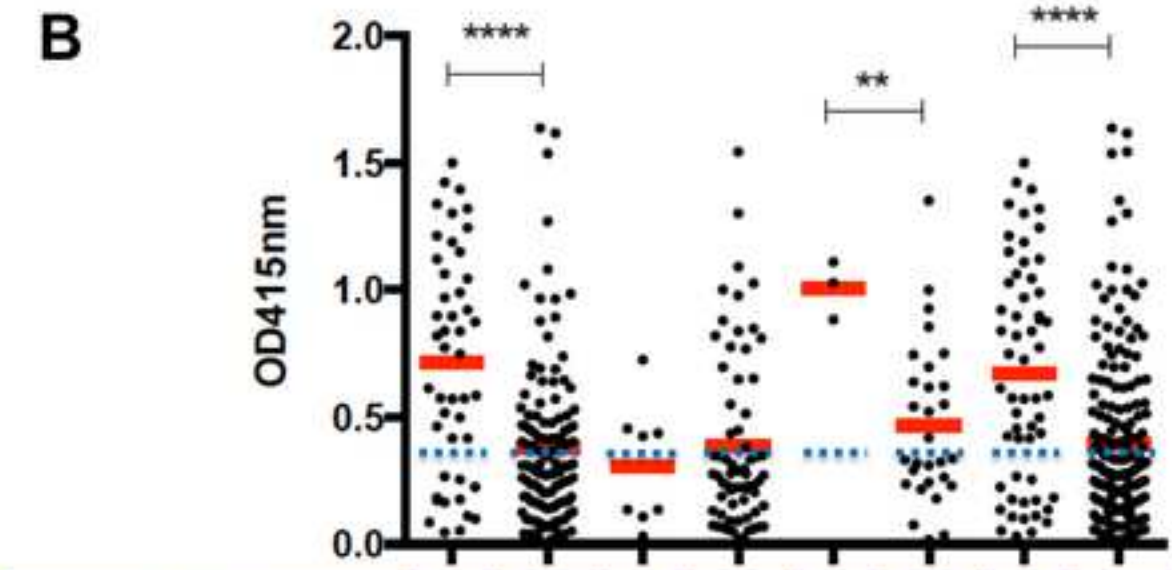


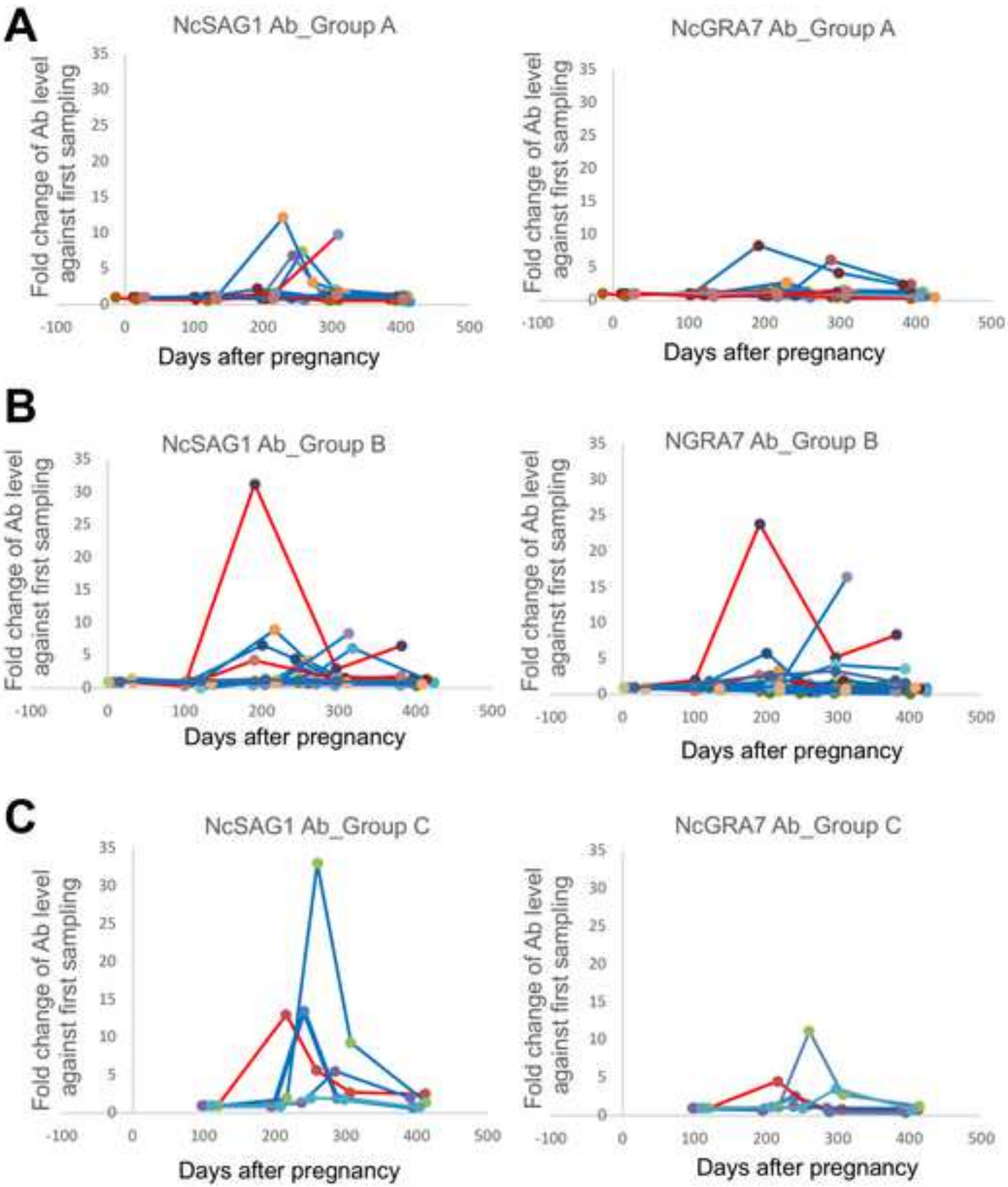
Figure 3



Abortion	+	-	+	-	+	-	+	-
Sample number	46	125	8	66	3	29	57	220
Seropositive rates	97.8%	85.6%	87.5%	68.2%	100%	84.2%	96.5%	76.3%
Category	Lactation		Dry		First pregnancy		Total	



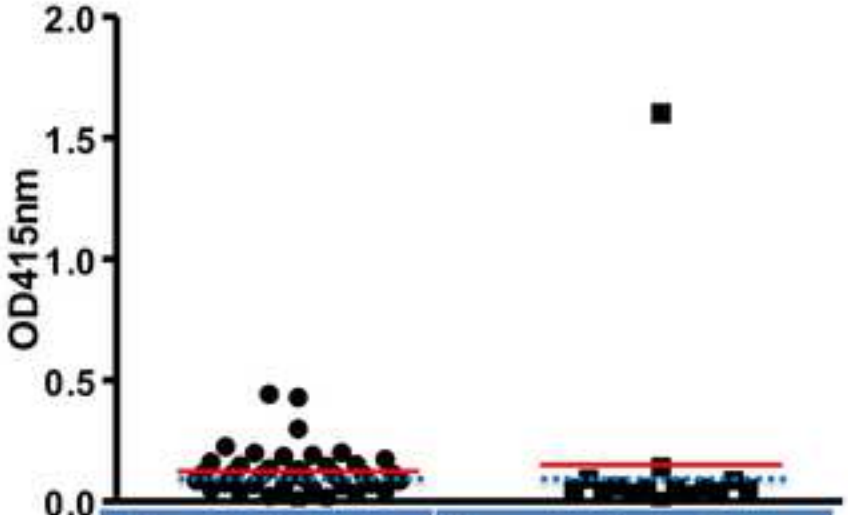
Abortion	+	-	+	-	+	-	+	-
Sample number	46	125	8	66	3	29	57	220
Seropositive rates	73.9%	57.6%	50%	50%	100%	69%	71.9%	53.6%
Category	Lactation		Dry		First pregnancy		Total	



Group	History of abortion [a]	*Neospora* Ab at first sampling [b]	Sample number
A	No	Negative	12
B	No	positive	19
C	Yes	positive	5

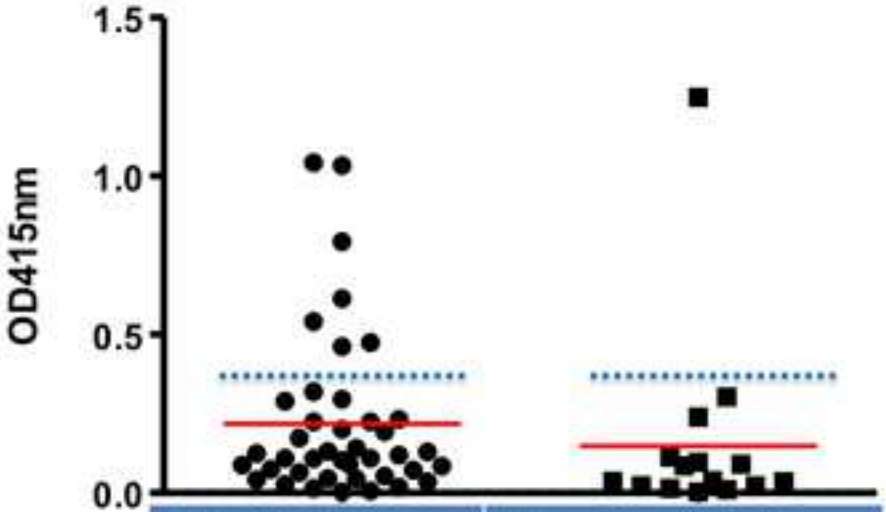
Figure 5

A



Neurological case		Non-neurologically case	
Total	positive (%)	Total	positive (%)
41	20 (48.8%)	16	2 (12.5%)

B



Neurological case		Non-neurological case	
Total	positive (%)	Total	positive (%)
41	7 (17%)	16	1 (6.25%)