

## Prevalence and Dynamics of Antibodies against NcSAG1 and NcGRA7 Antigens of *Neospora caninum* in Cattle during the Gestation Period

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**ABSTRACT.** Bovine abortion caused by the Apicomplexan parasite *Neospora caninum* is a major economic problem in the livestock industry worldwide. Our study measured the prevalence and temporal changes in levels of antibodies specific for two *N. caninum* derived antigens, NcSAG1 and NcGRA7, to determine an appropriate strategy for serodiagnosis. Using an enzyme-linked immunosorbent assay (ELISA), blood samples showed that 71 cows out of 129 were positive for anti-NcSAG1 antibodies and that only nine cows were positive for anti-NcGRA7 antibodies. By longitudinal sampling, it was revealed that positive and negative antibody conversion occurred frequently for anti-NcGRA7, but that anti-NcSAG1 antibodies persisted for a long-term. These results indicate the usefulness of measuring anti-NcSAG1 antibody levels for the detection of chronically infected cows. Twelve cows showed positive seroconversion during pregnancy, nine of which showed seropositivity for anti-NcGRA7 antibody at the sixth and/or seventh month of pregnancy; serum samples were not obtained from the remaining three cows during this period. Therefore, the optimal time for detection of anti-NcGRA7 antibodies appears to be between the fifth and eighth month of pregnancy.

**KEY WORDS:** ELISA, NcGRA7, NcSAG1, *Neospora caninum*.

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*Neospora caninum* is an intracellular Apicomplexan protozoan parasite similar to *Toxoplasma gondii* [4]. Neosporosis, the disease caused by *N. caninum*, is mainly observed in dogs and cattle. Canine neosporosis causes neuromuscular paralysis [12], while bovine neosporosis causes fetal abortion and neonatal mortality [6]. Failure of reproduction associated with *N. caninum* is a major economic problem in the livestock industry [6]. If naïve cattle are infected by oocysts shed by definitive hosts, such as dogs [10] or coyotes [7], sporozoites in the oocyst differentiate to tachyzoites and spread through the body of the cow. Parasites can exist over long periods as quiescent tissue cysts contained within the host tissue [6].

In the case of human abortion associated with *T. gondii*, a closely related parasite, transmission of the parasite to the placenta occurs predominantly in women who are primarily infected during the gestation period [11]. Congenital transmission of *T. gondii* in chronically infected women is very rare. In contrast to human toxoplasmosis, quiescent tissue cysts of *N. caninum* in latently infected cows are reactivated during pregnancy and cause reproduction failure [15]. For the control of cattle neosporosis, it is therefore important to detect and eliminate chronically infected cows from the

cattle herd.

To detect latent infection of *N. caninum* in cattle, an enzyme-linked immunosorbent assay (ELISA) using recombinant antigens derived from *N. caninum* has been developed as a highly specific and sensitive method for serodiagnosis [5]. This is especially the case for the *N. caninum* surface antigen NcSAG1 and the dense granule protein NcGRA7 antigen [1, 3, 9]. NcSAG1 is expressed in the tachyzoite and downregulated during the conversion from tachyzoite to bradyzoite [14]. The NcGRA7 protein is an immunodominant antigen shared by both tachyzoites and bradyzoites [2, 13]. It has been reported that titers of anti-NcGRA7 antibody in cows with a history of abortion are significantly higher than in non-aborting cows that were infected with *N. caninum* [9]. In addition, the frequency of anti-NcGRA7 antibody-positive individuals was higher among cows with a recent history of *N. caninum*-associated abortion than among cows that had experienced *N. caninum*-associated abortion more than 30 days previously [8]. In contrast, the frequency of anti-NcSAG1 antibody-positive individuals was similar between the two groups [8]. These phenomena might reflect the fact that anti-NcGRA7 antibodies are observed only during acute infection, including those infections arising out of recrudescence from a latent infection [1]. Serodiagnosis by ELISA with NcGRA7 antigens has potential as a method to estimate abortion risk associated with *N. caninum* infection. However, it is difficult to detect a temporal increase in anti-NcGRA7 antibodies during pregnancy by a single examination without knowing the time course of seroconversion. For practical use of ELISA using NcGRA7 antigen,

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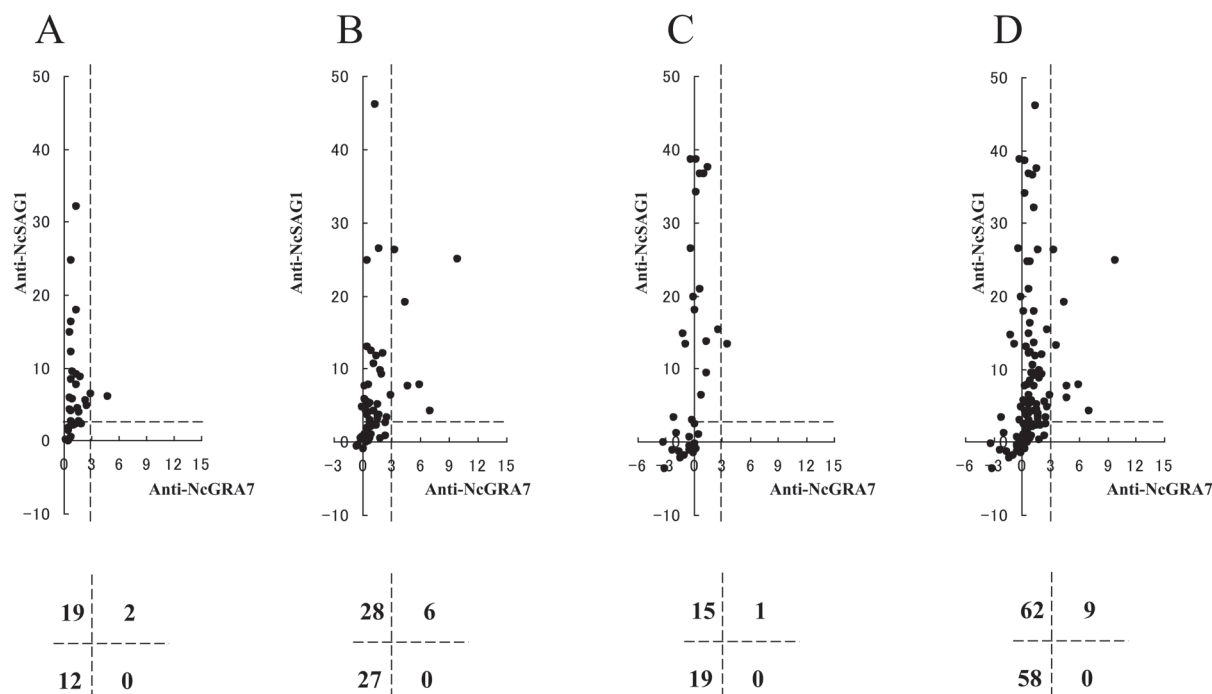


Fig. 1. (A, B, C) Relationships between anti-NcSAG1 and anti-NcGRA7 antibody levels in individual cows from three herds A, B and C. (D) Data for a total of 129 cows in A, B and C are shown. Vertical and horizontal lines indicate standardized values of anti-NcSAG1 and anti-NcGRA7 antibodies, respectively. Dashed lines indicate the positive boundary value (3.00) of the ELISA. Numeric characters inserted below indicate individual numbers in each area of the upper dot-blot graph.

it is necessary to determine the timing of positive conversion for anti-NcGRA7 antibody. In this study, we examined frequency and dynamics of serological reactions to NcSAG1 and NcGRA7.

## MATERIALS AND METHODS

**Serum samples:** A total of 129 cow serum samples were obtained from three herds, A, B and C (n=33, 61 and 35 cows, respectively), in Gifu Prefecture, Japan, in February 2009. To measure the time-dependent changes in antibody titers, repeat serum samples were obtained each month from a total of 29 individuals of herd C. Serum samples of 28 cattle were collected each month from July 2009 to March 2010 or until the cattle were removed from the farms. Blood samples of another cow were collected from December 2009 to March 2010. The 29 cows were separated into two groups; eighteen cows showed positive for anti-NcSAG1 antibody at the first blood sampling (Group I) and eleven cows were negative (Group II). In the sample collection period, 9 cattle in herd C showed seroconversion for anti-NcGRA7 antibody during gestation. Serum samples of three pregnant cattle in herd A were also collected from April 2012 to October 2012. Two of the three cattle delivered during the sampling period. Serum samples of the newborn calves and dams in herd A were also collected immediately after delivery but before the newborn calves suckled colostrums. These samples were used for analyzing dynamics of anti-NcGRA7 antibody

during gestation and estimating vertical transmission of the parasite to newborn calves. All blood samples were collected from the tail vein and centrifuged at  $1,000 \times g$  for 10 min before the serum was collected and stored at  $-20^{\circ}\text{C}$  for later use. All animal experiments were approved by the animal research committee of the Faculty of Applied Biological Science, Gifu University.

**Measurement of *N. caninum*-specific antibodies by ELISA:** Measurement of *N. caninum*-specific antibodies by ELISA was carried out as described previously [8]. Briefly, NcSAG1 and NcGRA7 recombinant proteins were expressed in *Escherichia coli* as glutathione S-transferase (GST) fusion proteins. Fifty microliters of purified rNcSAG1, rNcGRA7 and their control, GST, at a final concentration of  $0.1 \mu\text{M}$  were coated onto ELISA plates. After blocking with PBS containing 3% skim milk (PBSSM),  $50 \mu\text{l}$  of each serum sample diluted to 1:250 with PBSSM was added to duplicate wells, and the ELISA plate was incubated for 1 hr at  $37^{\circ}\text{C}$ . After washing with PBS containing 0.05% Tween-20, horseradish peroxidase conjugated goat anti-bovine total IgG (Bethyl Laboratories, Montgomery, TX, U.S.A.) diluted to 1:10,000 with PBSSM was added to each well and incubated at  $37^{\circ}\text{C}$  for 1 hr. After washing,  $100 \mu\text{l}$  2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonicsulphonic acid) substrate solution was added to each well. The absorbance at 415 nm was read after 1 hr of incubation at room temperature. Absorbance values (Abs) were determined as the difference in the mean optical density measured at 415 nm (OD415 nm) between the

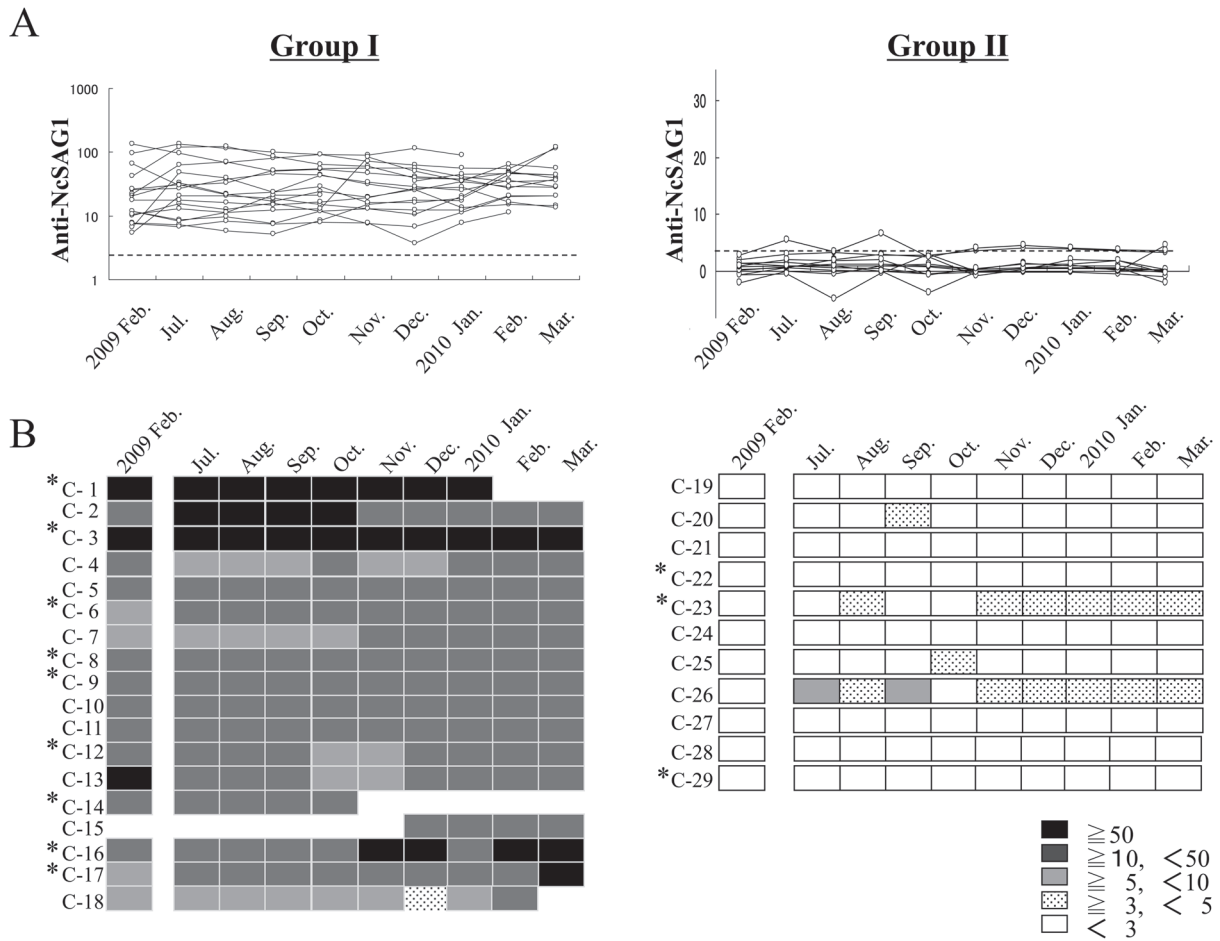


Fig. 2. Dynamics of anti-NcSAG1 antibody levels in cows from herd C, (Groups I and II). (A) Time-dependent changes in the anti-NcSAG1 antibody levels of cows. The vertical line indicates standardized values of anti-NcSAG1 antibody titer. The Dashed line indicates the positive boundary value (3.00) of the ELISA. (B) Standardized values of anti-NcSAG1 antibody titer in each individual were summarized. The color of each box indicates the antibody titer according to the inserted legend symbols. An asterisk (\*) indicates individuals that showed seroconversion for anti-NcGRA7 antibody, as shown in Fig. 3.

recombinant antigen (NcSAG1 or NcGRA7) and the GST protein. As an internal control, the OD415nm of standard *Neospora*-negative sera produced by our laboratory (n=9) [8] was also measured for each ELISA plate. Absorbance values of samples were standardized by the following formula:

Standardized values=(Abs of sample serum – average of Abs of negative sera)/standard deviation of Abs of negative sera.

When the standardized values were more than 3.00, the sample was considered positive.

**Statistic analysis:** Pearson’s product-moment correlation coefficient was calculated to assess the correlation between anti-NcSAG1 and anti-NcGRA7 antibody titers.

**RESULTS**

**Prevalence of anti-NcSAG1 and anti-NcGRA7 antibodies:** To investigate the frequency of anti-NcGRA7 and/or anti-NcSAG1 antibody-positive individuals, we examined serum

samples of 129 cows selected from three herds, A, B and C (n=33, 61 and 35, respectively). In herds A, B and C, 63.6% (21/33), 55.7% (34/61) and 45.7% (16/35) of cows were positive for anti-NcSAG1 antibody, respectively. On the other hand, 6.1% (2/33), 9.8% (6/61) and 2.9% (1/35) were positive for anti-NcGRA7 antibodies. All anti-NcGRA7 antibody-positive cows were also anti-NcSAG1 antibody-positive (Fig. 1). In total, 7.0% (9/129) of cows were double positive for anti-NcSAG1 and anti-NcGRA7 antibodies, 48.1% (62/129) were double negative for anti-NcSAG1 and anti-NcGRA7 antibodies, and 45.0% (58/129) were anti-NcSAG1 and anti-NcGRA7 antibody double-negative. No cows were identified as anti-NcSAG1 antibody negative and anti-NcGRA7 antibody positive (Fig. 1). No clear correlation was observed between anti-NcSAG1 and anti-NcGRA7 antibody levels. The correlation coefficients between anti-NcSAG1 and anti-NcGRA7 antibody levels in herds A, B and C were –0.0081 ( $P>0.05$ ), 0.37 ( $P<0.01$ ) and 0.44 ( $P<0.05$ ), respectively.

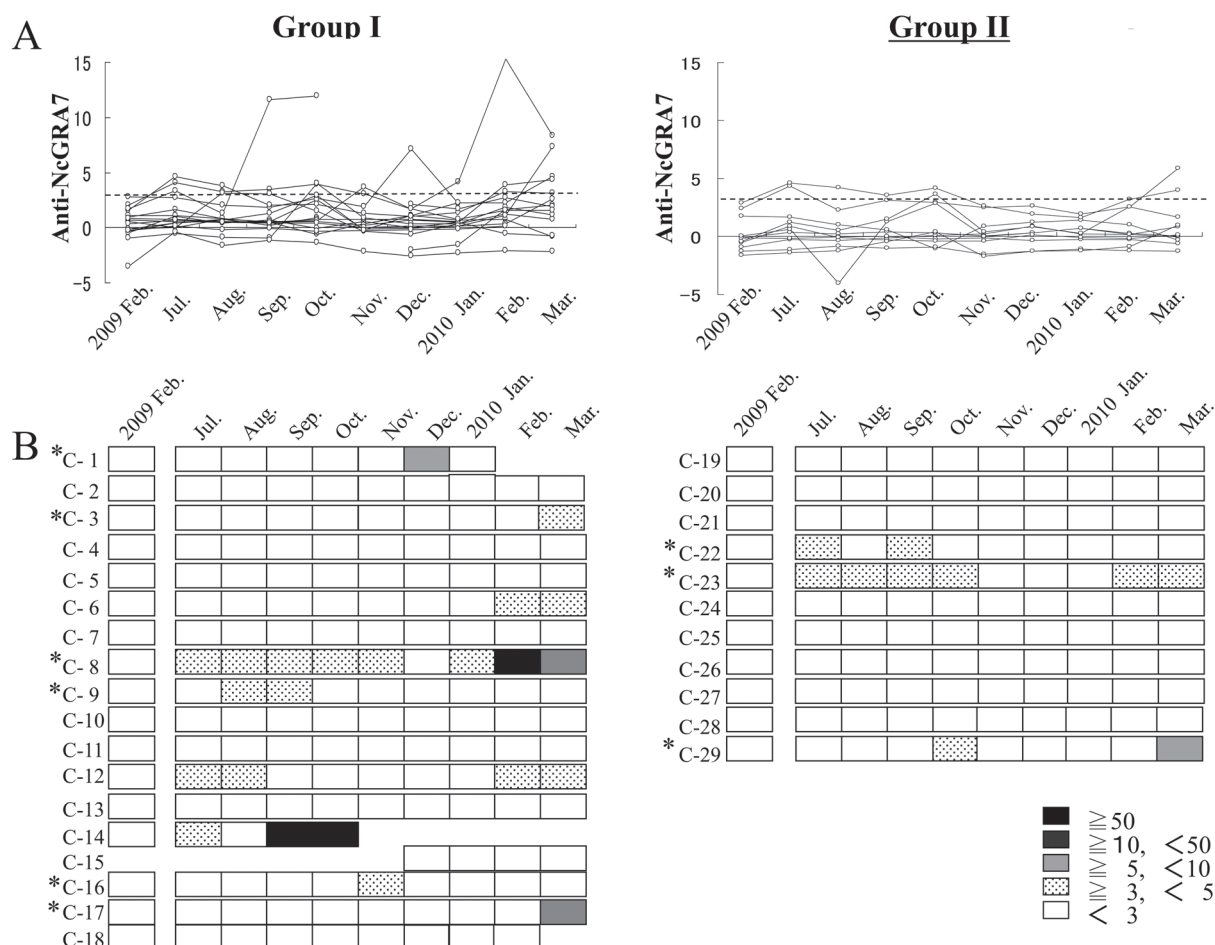


Fig. 3. Dynamics of anti-NcGRA7 antibody levels in cows from herd C, (Groups I and II). (A) Time-dependent changes in the anti-NcGRA7 antibody levels of cows. The vertical line indicates standardized values of anti-NcGRA7 antibody titer. The dashed line indicates the positive boundary value (3.00) of the ELISA. (B) Standardized values of anti-NcGRA7 antibody titer in each individual are summarized. The color of each box indicates the antibody titer according to the inserted legend symbols. An asterisk (\*) indicates individuals that showed seroconversion for anti-NcGRA7 antibody during gestation, as shown in Fig. 4.

*Dynamics of Anti-NcSAG1 and anti-NcGRA7 antibodies:* To investigate the dynamics of anti-NcSAG1 and NcGRA7 antibodies, serum samples were collected monthly from 29 cows in herd C, and the time-dependent changes in anti-NcGRA7 and anti-NcSAG1 antibody levels were observed between February 2009 and March 2010. Eighteen of 29 cows were positive for anti-NcSAG1 antibody at the first blood sampling (February 2009 for 17 cows and December 2009 for one cow) (Group I), and eleven cows were negative (February 2009 for all eleven cows) (Group II). All 18 cows in group I maintained anti-NcSAG1 seropositivity throughout the investigated period (February 2009 to March 2010) (Fig. 2). Although the anti-NcSAG1 antibody levels of 4 cows in Group II slightly exceeded the cutoff point during the study, a large increase in antibody levels was not observed in any cow in Group II (Fig. 2). Seven other cows in Group II maintained a negative status for anti-NcSAG1 antibodies throughout the investigated period (Fig. 2). These results indicated that the anti-NcSAG1

antibody levels of each individual were constant, regardless of the timing of the sampling.

In contrast, as shown in Fig. 3, anti-NcGRA7 antibody levels of cows from Group I were drastically changed during the investigated period. At the time of the first blood sampling, only one cow in Group I was positive for anti-NcGRA7 antibodies. However, nine cows in Group I showed seroconversion to anti-NcGRA7 antibodies at some point during the investigated period (Fig. 2). Cows in group II were negative for anti-NcGRA7 antibodies during the majority period of the investigation, except for three cows that displayed a very slight excess of anti-NcGRA7 above the cutoff point for a short period (Fig. 2). Clear seasonality of the seroconversion was not observed in either Group I or II (Fig. 2).

*Dynamics of anti-NcGRA7 antibody during gestation:* Monthly collected blood samples from 9 cows that showed seroconversion for anti-NcGRA7 antibody during gesta-

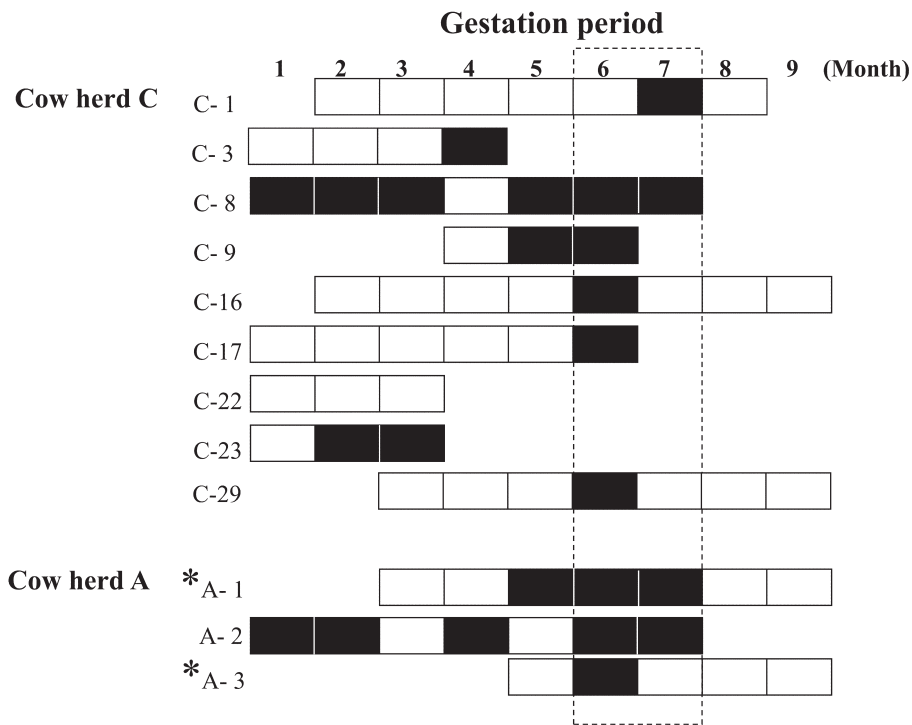


Fig. 4. Dynamics of anti-NcGRA7 antibody levels during gestation. Time-dependent changes in anti-NcGRA7 antibody levels in 9 cows from herd C and three cows from herd A are shown. Black and white boxes indicate anti-NcGRA7 antibody positive and negative, respectively. Asterisks (\* and \*\*) indicate individuals whose newborn calves were examined, as shown in Fig. 5.

tion were analyzed to investigate the relationship between the term of pregnancy and the dynamics of anti-NcGRA7 antibodies. As shown in Fig. 4, all the cows studied, except for the three cows from which serum could not be collected during the middle gestation period, showed seroconversion at the sixth and/or seventh month of pregnancy. Twelve cows did not show any abortion or stillbirth during the study.

*Levels of anti-NcSAG1 and anti-NcGRA7 antibodies of newborn calves:* To examine the possibility that the seroconversion for anti-NcGRA7 antibody during the sixth and/or seventh month of pregnancy reflected the recrudesence of latent parasites, we collected sera of 2 newborn calves in herd A (dams are shown as A-1 and A-3 in Fig. 3). In both cases, the sera of the newborn calves contained a high level of anti-NcGRA7 antibody even before suckling any colostrum (Fig. 5). It is likely that the seroconversion of dams during the sixth and/or seventh month of pregnancy reflected the recrudesence of latent infection. In contrast to the newborn calves, both A-1 and A-3 dams were negative for anti-NcGRA7 antibody in the calving month (Figs. 4 and 5).

DISCUSSION

The detection and elimination of chronically infected cows is important for preventing the endogenous vertical transmission of *N. caninum*. At the same time, to evaluate abortion risk caused by *N. caninum*, it is important to

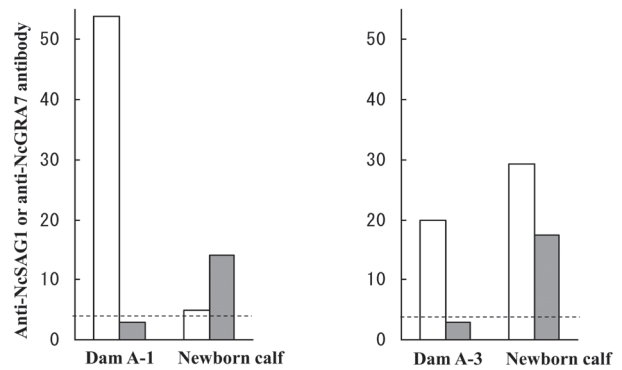


Fig. 5. Levels of anti-NcSAG1 and anti-NcGRA7 antibodies of dams A-1 and A-3 and their newborn calves (white and gray bars, respectively). The vertical line indicates standardized values of anti-NcSAG1 and anti-NcGRA7 antibody titers. The dashed line indicates the positive boundary value (3.00) of the ELISA. Serum samples of dams and newborn calves were collected just after birth and before suckling colostrums.

discriminate between active and latent infection. To determine appropriate deployment of ELISA using relatively well-studied antigens, NcSAG1 and NcGRA7, for the diagnosis of cattle neosporosis, we investigated the prevalence and dynamics of antibodies against these antigens. In our

study, only a few cows were positive for anti-NcGRA7 antibodies, while 55% of cows (71/129) were positive for anti-NcSAG1 antibody. In addition, consistent with previous studies [8], anti-NcSAG1 antibody levels of each individual were constant regardless of the timing of the examination. Considering that the anti-NcGRA7 antibody level increases during acute infection [1], it is likely that the majority of anti-NcSAG1-positive cows in the herds were in the latent phase. This suggests that ELISA using anti-NcSAG1 antigen is a promising tool for detecting latently-infected cattle and that examinations do not have to be too frequent, e.g., once a year. In contrast, levels of anti-NcGRA7 antibodies varied greatly over time, and a one-off ELISA test would not be efficient for detecting anti-NcGRA7 antibody within a herd. The usefulness of anti-NcGRA7 antibodies as a marker of abortion risk has been reported previously [9], but repeated analysis at frequent intervals will be necessary to detect and measure anti-NcGRA7 antibody accurately. In this study, we showed that positive conversion of anti-NcGRA7 antibody occurred at the sixth and/or seventh month of gestation. These results strongly suggest that recrudescence from latent cysts does not occur in a random manner, but occurs specifically from the sixth to seventh month of gestation. This period must therefore be the optimal time point for the detection of anti-NcGRA7 antibodies.

During this study, no cow showed clinical symptoms associated with neosporosis including abortion and stillbirth. However, anti-NcGRA7 antibody titer increases during acute infection [1]. The temporal increase in anti-NcGRA7 antibody levels during gestation therefore suggest recrudescence of latently infected parasites without obvious symptoms in the dam. In addition, we showed that newborn calves born from such dams with anti-NcGRA7 antibodies were serologically positive to the same antigen before suckling colostrums. Considering that maternal antibody does not transfer to the fetus through the ruminant placenta, the results indicate that both of the newborn calves were infected in the uterus and that the antibodies were being produced by the fetus. It is strongly suggested that vertical transmission occurred to newborn calves from dams that had shown positive conversion for the anti-NcGRA7 antibody during gestation. An increase in anti-NcGRA7 antibody levels during gestation can be a marker for recrudescence of the parasite. Such vertical transmission is a severe concern for the spread of the parasite, because congenitally infected calves remain persistently infected and can pass the infection on to the next generation [5]. Repeated examination, particularly during the sixth and seventh month of pregnancy, by ELISA using the NcGRA7 antigen can be a useful diagnostic tool to detect asymptomatic congenital infection with *N. caninum*.

In this study, we described the dynamics of anti-NcSAG1 and anti-NcGRA7 antibodies in cattle with special reference to changes during the gestation period. Measurement of antibodies against these two *N. caninum* antigens could be a valuable diagnostic tool for the control of cattle neosporosis.

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