An ELISA using recombinant NcGRA7 could detect *Neospora caninum* infection in aborting cows

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ネオスポラ原虫感染による流産牛を検出可能な組換えNcGRA7タンパク質を抗原としたELISA法の確立

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1. Introduction

Neospora caninum, is an intracellular apicomplexan parasite that causes stillbirth and abortion in cattle and neuromuscular disorders in dogs (8). In 1984, a neuroloagical disease was recognized in dogs in Norway that appeared to be associated with a parasite similar to *Toxoplasma gondii* (4). However, *T. gondii* antibody was not found in the sera of the dogs. The subsequent characterization and classification in 1988 by Dubey and co-workers further confirmed that this parasite is really different from *T. gondii* (10). Thus, the parasite was given a name as *N. caninum* and classified into the same phylum Apicomplexa as *T. gondii*, but different species.

Domestic dogs are the known definitive host for *N. caninum*, and, recently (19), coyotes were also demonstrated as final hosts of *N. caninum* (14). The life cycle of *N. caninum* is typified by 3 infectious stages: tachyzoites, tissue cysts, and oocysts. Tachyzoites can be vertically transmitted from a persistently infected mother to her fetus (6), and horizontal transmission could also occur by the uptake of sporulated oocysts shed by the definitive hosts or tissue cysts (13). Vertical transmission and horizontal transmission increase the possibility of the wide prevalence of neosporosis.

N. caninum infection has been reported in various animals, including cattle, goats, sheep, horses, deer, foxes, dingoes, raccoons, and coyotes (7). Current evidence strongly suggests that infection with *N. caninum* represents a major cause of reproductive failure and abortion in cattle,

resulting in alarming economic losses to the livestock industry worldwide (9). Infected cows at any age may abort from 3 months of gestation to term, and most abortions occur at 5-6 months of gestation (7). Fetuses may die in utero, be resorbed, mummified, autolyzed, stillborn, born alive with clinical signs, or born clinically normal but persistently infected (2, 25). Both dairy and beef cattle with antibodies to *N. caninum* (seropositive) are more likely to abort than seronegative cows and up to 95% of live born calves from seropositive dams will be congenitally infected and clinically normal (25). Quantitative studies in the USA, New Zealand, the Netherlands, and Germany have indicated that 12 to 42% of aborted fetuses from dairy cattle were infected with *N. caninum* and that up to 90% of cattle in some herds were infected (7).

Many diagnostic methods based on histopathology, demonstration of the pathogen, immunology and polymerase chain reaction (PCR), have been developed to determine *N. caninum* infection in animals and bovine abortion associated with *N. caninum* infection (9). Although a definitive diagnosis of bovine abortion caused by *N. caninum* is needed to demonstrate that there are parasites in the lesions and exclude other causes of abortion, serologic diagnosis, such as that with the enzyme-linked immunosorbent assay (ELISA), is important and widely used. A number of antigens have been evaluated as potential diagnosis antigens for the detection of an antibody to *N. caninum*, including tachyzoite lysates and recombinant proteins. Recently, ELISA with recombinant antigen has been widely evaluated (9), and in the future it will become more

important since the recombinant antigens can be produced easier in large quantities and better standardized for the production of serological assays. The surface proteins NcSAG1 and NcSRS2 (17) and the dense-granule protein NcGRA7 (1) are three important immunodominant proteins of *N. caninum*. The recombinant proteins of NcSAG1, NcSRS2 and NcGRA7 have been used for the serologic diagnosis of *N. caninum* infection (9). Although *N. caninum* has been found to be a major cause of bovine abortion, a marker for the serodiagnosis of *Neospora* infection in an aborting cows has not been identified. In this study, we compared ELISAs based on the recombinant antigens NcSAG1, NcSRS2, and NcGRA7 and the *N. caninum* tachyzoite lysate antigen (NLA) for the detection of the *N. caninum*-specific antibody in the aborting cows, and search for a new marker among these proteins for the serodiagnosis of *Neospora* infection in abortion cows.

2. Materials and methods

2.1. Parasite

Tachyzoites of the *N. caninum* Nc-1 strain (Kindly provided by Dr. J. P. Dubey, United States Department of Agriculture, Agriculture Research Service, Livestock and Poultry Sciences Institute, and Parasite Biology and Epidemiology Laboratory) were cultured in Vero cell monolayers in a minimum essential medium supplemented with 8% fetal bovine serum and kanamycin (50 μg/ml) at 37°C in a 5% CO_2 air environment. For the purification of tachyzoites, the parasites were scraped from the flask 4 days

post infection and then passed through a 27G needle and a 5.0 μm filter (Millipore, USA). They were then washed in phosphate-buffered saline (PBS). The pellet was stored at –30°C until use.

2.2. Cloning of the NcGRA7, NcSAG1 and NcSRS2 genes

The *N. caninum* parasite (1×10^8) were lysed in 0.1M Tris-HCL (pH□ 8.0) containing 1% sodium dodecyl sulfate, 0.1M NaCl, and 10 mM EDTA and then digested with proteinase K (100 μg/ml) for 2 hrs at 55°C. The DNA was extracted with phenol/chloroform and precipitated by ethanol. The pellet was dissolved in a TE buffer (10mM Tris-HCL, pH□8.0, 1mM EDTA) and used as template DNA for a polymerase chain reaction (PCR). Three primer sets (Table 2) were used to amplify three genes encoding NcSAG1, NcSRS2, NcGRA7, respectively. Then the genes were clone to pGEX expression serial vectors (5, 16, 12). The resulting plasmid DNA containing NcSAG1, NcSRS2 and NcGRA7 genes (designed as pGEX/NcSAG1, pGEX/NcSRS2, pGEX/NcGRA7) were transformed to BL21 *Escherichia coli* for expression of recombinant proteins.

2.3. Expression and purification of recombinant protein

Escherichia coli (*E. coli*) transformed with the pGEX/NcSAG1, pGEX/NcSRS2, pGEX/NcGRA7 was cultured in LB medium (1% tryptone peptone, 0.5% yeast extract, 1% NaCl) with ampicillin (50 μg/ml) at 37°C with vigorous shaking. When the absorbance of the culture reached

0.3~0.5 at $OD_{600\ nm}$ isopropyl β-D-thiogalactopyranoside (0.5mM) was added to induce the expression of NcSAG1, NcSRS2, NcGRA7 as a fusion protein with glutathione-*S*-transferase (GST). After additional incubation for 4 hrs, *E. coli* was harvested by centrifugation and treated by sonication in a TNE buffer (50mM Tris-HCl, pH, 7.5, 2mM EDTA, 0.1M NaCl) containing lysozyme (100 μg/ml) and 1% Triton X-100, which was then incubated at room temperature for 30 minutes. The cell debris were separated and supernatant was collected by centrifugation. The fusion proteins (designated as NcSAG1, NcSRS2 and NcGRA7) were purified using glutathione sepharose 4B beads based on the manufacturer's instructions (Amersham Pharmacia Biotech, USA). GST protein was also purified from *E. coli* transformed with plasmid pGEX without any insert DNA as control. The protein concentration was determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) analysis using BSA standards. SDS-PAGE was performed under standard conditions.

2.4. Preparation of tachyzoite lysates

For preparation of *N. caninum* tachyzoites lysate antigen(NLA), purified tachyzoites (1×10^8) were suspended in 300 μl PBS and sonicated on ice 6 times, 30 seconds for each time with 30 seconds interval. Subsequently, the sonicated-suspension was centrifuged at 12,000 × *g* for 30 min at 4°C. The supernatant was collected and its concentration was determined by a

BCA kit (Pierce, USA) based on the manufacturer's instructions. All the antigens were stored at -30°C until use.

2.5. ELISA

Fifty microliter of purified NcSAG1, NcSRS2, NcGRA7 and their control GST as well as NLA at a final concentration of 5 μg/ml were coated to the ELISA plates (Nunc, Denmark) overnight at 4°C with a carbonate-bicarbonate buffer (pH, 9.6). After blocking with PBS containing 3% skim milk (PBS-SM) for 1 hr at 37°C, the plates were washed twice with PBS containing 0.05% Tween20 (PBS-T), and 100 μl of serum samples diluted at 1:250 with PBS-SM was added to duplicate wells. Plates were incubated at 37°C for 1 hr. After washing five times with PBS-T, plates were incubated with horseradish peroxidase-conjugated goat anti-bovine IgG+IgA+IgM (Bethyl laboratories, USA) diluted at 1:4,000 with PBS-SM at 37°C for 1 hr.

The plates were washed five times, and then substrate solution (0.1 M citric acid, 0.2M sodium phosphate, 0.003% H_2O_2, and 0.3 mg/ml 2,2′ 2 -azide-bis [3-ethylbenzthiazoline-6-sulfonic acid]; Sigma, USA) was added to each well in 100μl aliquots. The absorbance at 415 nm was read after 1 hr of incubation at room temperature by using an ELISA reader (Corona Microplate Reader MTP-120; Corona, Japan). The ELISA result was determined by the difference in the mean optical density at 415 nm ($OD_{415\ nm}$) value between that of the recombinant antigens (NcSGA1, NcSRS2 or NcGRA7) and that of GST protein. For ELISA using NLA, the result was

determined by just taking the $OD_{415\,nm}$ value. The cutoff point of 0.04 was determined as the $OD_{415\,nm}$ value for neospora negative sera plus three standard deviations.

2.6. Sera

Bovine serum samples (n=62) provided by Souya Livestock Hygiene Service Center, Hokkaido, Japan, which were obtained from three Holstein dairy herds (A, B and C farms, Table 1) with a history of *Neospora*-associated abortions, were used in this study. All the sera are known seropositive to *N. caninum* confirmed by indirect fluorescent-antibody test (IFAT, sera with antibody titers of >1:200). In the case of abortion, fetal samples (cerebrum, cerebellum, liver, and skeletal muscle) were examined through an immunohistochemical test, and the tachyzoites were detected in the lesions. In addition, their mother's sera used in this study were seropositive to *N. caninum* by IFAT. The serum samples were classified into three groups (Table 1), i.e., group 1, 16 samples from aborting cows (gestation ranging from 3 to 7 months), group 2, 36 samples from non-aborting cows, and group 3, 10 samples from heifers. The ranges of period from abortion to sampling are 13 months, 0.3-8 months and 1-5 months in farms A, B and C, respectively.

2.7. Statistic analysis

Data were analyzed by ANOVA. The difference among the mean $OD_{415\,nm}$ was analyzed using Turkey-Kramer multiple comparison tests.

Pearson's correlation coefficient analysis and simple regression were used to assess the relationship between the $OD_{415\ nm}$ values from ELISA with two antigens. The difference in the correlation coefficients of the regression lines obtained from two groups was determined by testing the *t*-value. The results were though to be significant different if $p<0.05$.

3. Results

3.1. Purification of antigens for ELISA

In this study, four kinds of antigens were prepared for ELISA detection. As shown in Figure 1, three recombinant proteins NcSAG1, NcSRS2 and NcGRA7 were purified from the soluble fractions with Glitathione Sepharose 4B with molecular mass of 54 kDa, 61 kDa and 47 kDa. Tachyzoite lysate NLA was also obtained and shown in Figure 1.

3.2. Detection of antibody to *N. caninum* by ELISA

To detect the specific antibody associated with the parasite-induced abortion, bovine sera of the above three groups were examined by ELISA with four antigens, NcSAG1, NcSRS2, NcGRA7, and NLA (Fig. 2). Among the three serum groups, the mean values of $OD_{415\ nm}$ of group 1 were higher than those of group 2 and 3 in the ELISAs with recombinant antigens. The ELISA with recombinant antigens could discriminate between group 1 and group 3 ($P < 0.01$), while there was no statistically significant difference among the groups by the ELISA with NLA. These results indicated that the specific antibodies against NcSAG1, NcSRS2 and

NcGRA7 were produced in the aborting cows. However, in the ELISAs with NcSAG1 and NcSRS2, there was no statistically significant difference between aborting and non-aborting cows. More importantly, the ELISA with NcGRA7 could discriminate the aborting cows from the parasite-infected animals ($P<0.01$).

In addition, means value of $OD_{415\ nm}$ of each sample group in recombinant antigen ELISAs were found higher than the means of OD value by NLA ELISA, indicating that recombinant antigen-based ELISA is more sensitive than NLA ELISA in detection of antibody to *N. caninum*.

3.3. Comparison of antibody response among recombinant antigen-based ELISAs

In order to examine the distribution of $OD_{415\ nm}$ value between aborting and non-aborting cows, a further comparison of ELISAs with recombinant antigens was performed (Fig. 3). In Figure 3A, positive correlations were found between the $OD_{415\ nm}$ values of the ELISAs with NcSAG1 and NcSRS2 in both aborting cows ($r = 0.68$, $P<0.01$) and non-aborted cows ($r = 0.732$, $P<0.01$). However, when the difference in the correlation coefficients of the regression lines obtained from aborting and non-aborting cows was examined, no statistically significant difference was found. This result indicates that the pattern of antibody production against NcSAG1 and NcSRS2 were not different in aborting and non-aborting cows. Then tried to determine whether the antibody production against NcGRA7 and the other two molecules have a correlation among animals

(Fig. 3B and C). A simple regression analysis revealed a correlation between antibody responses against NcGRA7 and other recombinant antigens in aborting cows (NcGRA7 and NcSRS2, r = 0.663, $P<0.01$; NcGRA7 and NcSAG1, r = 0.719, $P<0.01$). In contrast, there was no correlation in the antibody responses from non-aborting cows. These results indicate that the production of the anti-NcGRA7 antibody is upregulated in the aborting cows.

4. Discussion

In the last decade, a large number of *N. caninum* proteins have been evaluated as a potential diagnostic antigen. The whole tachyzoite lysate has been used to detect the antibody to *N. caninum* in several serological diagnosis tests including commercialized tests for long time (9). However, recent years, recombinant antigens have been used widely due to its advantages in specificity, sensitivity, easy to produce in large quantity and easy to standardize for the production of serological assays. *N. caninum* and *T. gondii* are two close parasites, *N. caninum* tachyzoites lysate contains many proteins which cross-react with *T. gondii* antibody and the lysate may also contain host cell component during preparation of the lysate. These can easily cause non-specific reaction in the assays. Furthermore, preparation of the lysate by sonication may have resulted in the degradation/proteolysis of some proteins, which might reduce the reaction with certain antibody. In this study, the OD value detected by NLA is much lower than those detected by recombinant antigens. The

results strongly indicate that recombinant antigens have the potential to replace the antigen obtained from the lyzed whole parasites for ELISA detection.

Evidences have shown that aborted cattle due to neosporosis have higher *N. caninum*-specific antibody response than infected but non-aborting cattle (9). However, a previous study has shown that no serological test could be used to establish definitively that *N. caninum* caused an abortion in an individual cow (11). In order to estimate whether the production of an antigen-specific antibody is associated with abortion induced by *N. caninum* infection, 62 serum samples were classified into three groups, namely, aborting cows, non-aborting cows and heifers and subjected to ELISAs using NcSAG1, NcSRS2, NcGRA7, and the parasite lysates as antigens. Our results show that a higher level of antibodies against NcSAG1 and NcSRS2 was detectable in aborting cows than in heifers. The recombinant antigens NcSAG1 and NcSRS2 could not discriminate between aborting and non-aborting cows. Therefore, production of these antibodies might be induced in parasite-infected cows through their pregnancy. On the other hand, the levels of the anti-NcGRA7 antibody in aborting cows were significantly higher than those in non-aborting cows and heifers. Although the analysis about the time points of NcGRA7-specific antibody production during the pregnancy would be required in the further study, these data suggest that NcGRA7 might be a new marker for the serodiagnosis of *N. caninum* infection resulting in abortion in cows.

In cattle herds with endemic abortion due to neosporosis, there is often a positive association between the serostatus of mother and daughters (21, 23). There is evidence that the recrudescence of latent infection during gestation is responsible for an increased abortion risk (15, 20, 22). Moreover, a small proportion (less than 5%) of cows may have repeatedly abortion due to neosporosis (3). On the other hand, epidemiologic data has shown evidence for protective immunity to *N. caninum*-associated abortion when chronically infected dams are re-infected horizontally (18). This observation was confirmed when naturally infected dams received an experimental challenge infection (24). These reports make it difficult to understand why *N. caninum* infection could induce abortion. Present study suggested that NcSAG1 and NcSRS2 might be upregulated through the parasite infection and gestation and that NcGRA7 might play a role in parasite-induced abortion. Therefore, identification of parasite molecules specifically induced in the aborting cows might trigger an investigation the mechanism of parasite-associated abortion.

5. Summary

Neospora caninum is a major cause of abortion in cattle. In order to investigate whether the production of an antigen-specific antibody is associated with *Neopora*-induced bovine abortion, four ELISAs based on recombinant antigens NcSAG1, NcSRS2, NcGRA7 and *N. caninum* tachyzoites lysate (NLA) were used to detect *N. caninum* antibody in

aborting cows, non-aborting cows, and heifers which were all conformed seropositive to *N. caninum* in this study. The results show that the ELISAs with recombinant antigens could detect higher level of *N. canimun* antibody in aborting cows than those detected by ELISA with NLA, indicating that the specific antibodies against NcSAG1, NcSRS2 and NcGRA7 are produced in aborting cows. More importantly, the levels of the anti-NcGRA7 antibody in aborting cows were significantly higher than those in non-aborting cows and heifers, suggesting that NcGRA7 would be a new marker for the serodiagnosis of *N. caninum* infection resulting in abortion.

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Table 1. Sera from dairy farms infected with *N. caninum*.

Group	Clinical state	Farm A	Farm B	Farm C	Total
1	Aborting cows	7	4	5	16
2	Non-aborting cows	11	25	0	36
3	Heifers	5	5	0	10
Total		23	34	5	62

Table 2. Primer sets for gene cloning.

Gene	Primer	Size of amplified DNA (bp)
NcSAG1	Forward: 5′ -ACGAATTCATCAGAAAAATCACCT-3′	806
	Reverse: 5′ -ACGAATTCGACCAACATTTTCAGC-3′	
NcSRS2	Forward: 5′ -ACGAATTCTGCGCCGTTCAAGTCG-3′	987
	Forward: 5′ -ACGAATTCAAGGCAACTCGTCGTC-3′	
NcGRA7	Forward: 5′ -ACGAATTCTAAAATGGCCCGACAAG-3′	574
	Reverse: 5′ -ACGAATTCCTATTCGGTGTCTACTT-3′	

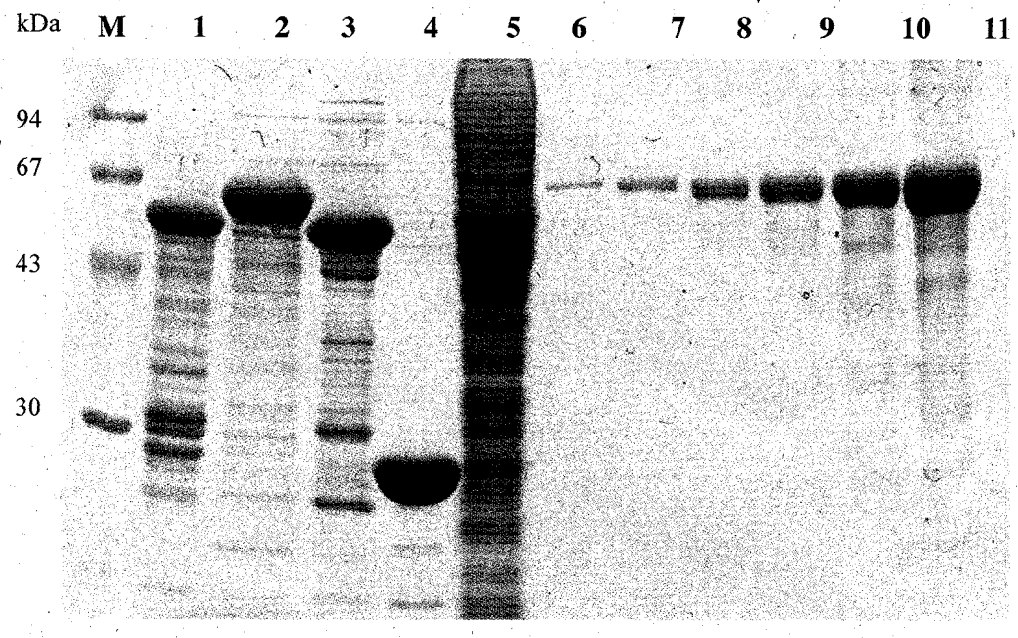

Fig. 1. The proteins used in ELISA. Recombinant proteins were purified from the soluble fraction of *E. coli* culture with Glutathione Sepharose 4B. The concentration of the recombinant proteins was determined by comparing the density of the protein bands with the density of BSA bands in various concentrations. *N. caninum* tachyzoite lysate was shown as well. Proteins were stained with Coomassie Brilliant Blue. M, standard molecular mass;, lane 1, NcSAG1/GST; lane 2, NcSRS2/GST; lane 3, NcGRA7/GST; lane 4, GST; lane 5, *N. caninum* tachyzoite lysate; lane 6-11, BSA standard with concentration at 62.5, 125, 250, 500,1000 and 2000 μg/ml, respectively.

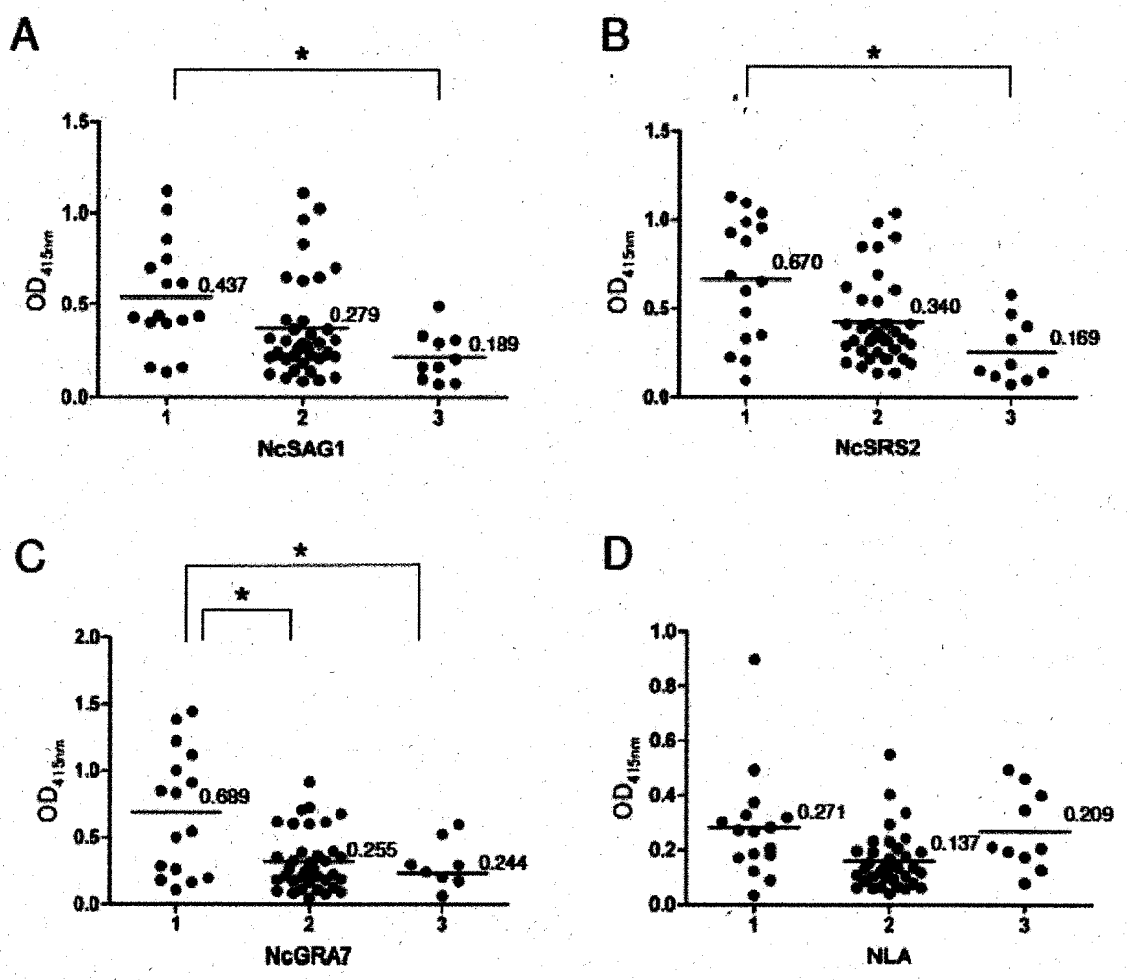

Fig. 2. Detection of antibody to *N. caninum* by ELISA with NcSAG1 (A), NcSRS2 (B), NcGRA7 (C) and the parasite lysates (NLA) (D). Group 1 indicates serum samples from aborting cows. Group 2 indicates samples from non-aborting cows. Group 3 indicates samples from heifers. The mean $OD_{415\ nm}$ values were shown. Data were analyzed by ANOVA, and then differences among mean $OD_{415\ nm}$ values were analyzed using Turkey-Kramer multiple comparison tests. (*) There is statistical significant difference among the samples ($P<0.05$). The $OD_{415\ nm}$ values were representative from at least three repeated experiments.

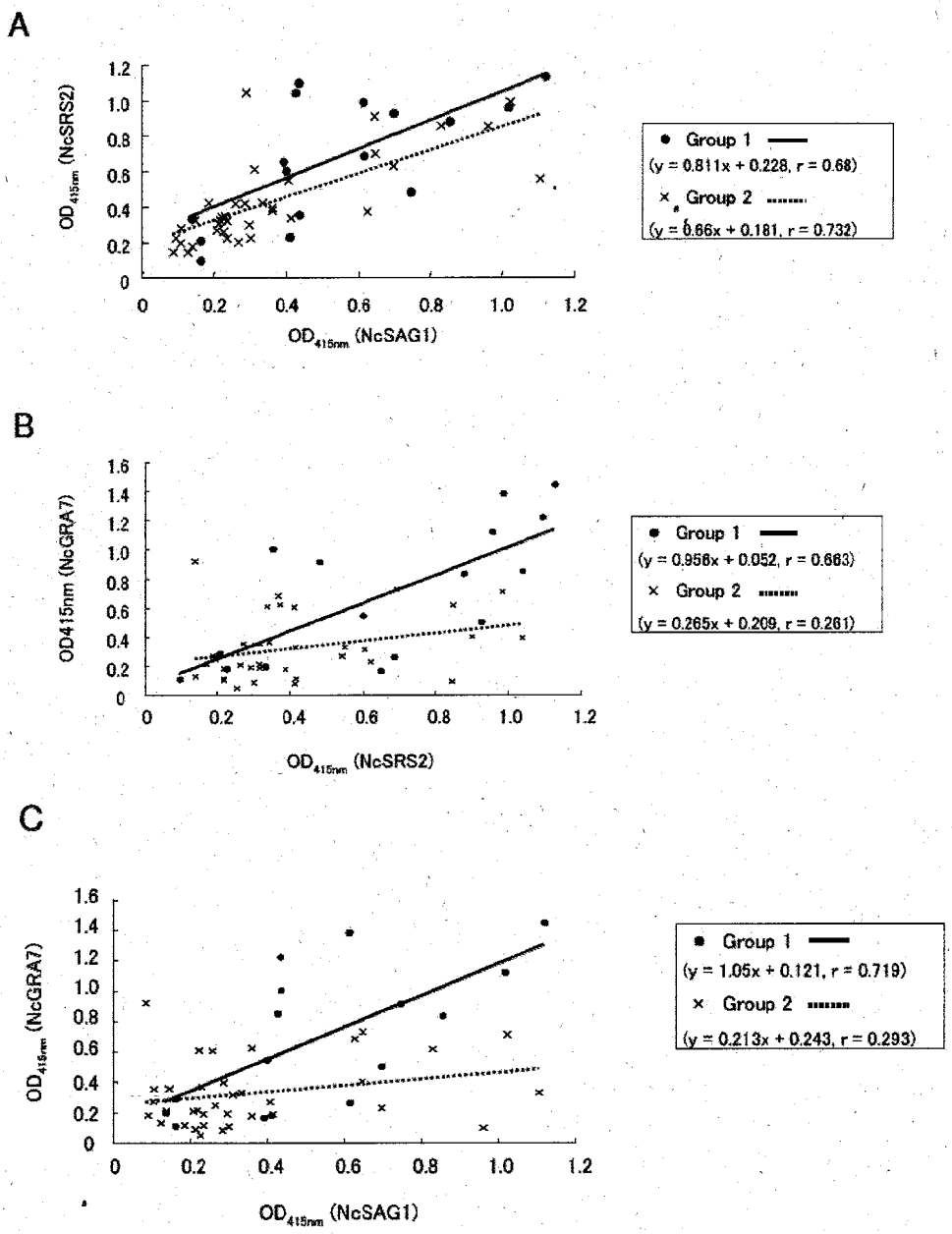

Fig. 3. Comparison of correlation between $OD_{415\ nm}$ values from ELISA with two antigens. (A) Correlation between $OD_{415\ nm}$ values from ELISA with NcSAG1 (x-ray) and NcSRS2 (y-ray). (B) Correlation between $OD_{415\ nm}$ values from ELISA with NcSRS2 (x-ray) and NcGRA7 (y-ray). (C) Correlation between $OD_{415\ nm}$ values from ELISA with NcSAG1 (x-ray) and NcGRA7 (y-ray). Group 1 indicates serum samples from aborting cows. Group 2 indicates samples from non-aborting cows. Peason's correlation coefficient analysis and simple regression were used to assess the relation between $OD_{415\ nm}$ values from ELISA with two antigens. Difference of correlation coefficients of the regression lines obtained from two groups was determined by testing the *t*-value.

ネオスポラ原虫感染による流産牛を検出可能な組換えNcGRA7タンパク質を抗原としたELISA法の確立

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「目的」 ネオスポラ原虫（*Neospora caninum*）は犬を終宿主とし、牛、羊、山羊などを中間宿主とする細胞内寄生性原虫である。終宿主の糞便中に排出されるオーシストによる水平感染や中間宿主における垂直感染により伝搬される。特に牛には流産、死産或いは子牛の神経症状を主徴とする異常産を高率に引き起こす。欧米諸国では牛の流産の約40%の原因はネオスポラ原虫感染によるものと報告されている。しかしながら、今のところネオスポラ原虫感染による流産牛を検出できる血清学診断法はまだ確立されていない。そこで、本研究ではネオスポラ原虫感染による流産牛を検出可能な血清学診断法を開発するために、3種類の組換え抗原を用いたELISA法を確立し、流産牛血清中のネオスポラ原虫特異抗体の検出を試みた。

「材料と方法」 16頭の流産牛血清、36頭の非流産牛血清、及び10頭の非妊娠牛（妊娠歴のない牛）血清を供試サンプルとした。ネオスポラ原虫の主要表面抗原であるNcSAG1、NcSRS2、及び主要分泌抗原であるNcGRA7の遺伝子をそれぞれ大腸菌にて発現・精製し、それぞれの組換えタンパク質を抗原としたELISA法を確立した。なお、対照としてネオスポラ原虫全虫体タンパク質を抗原としたELISA法を用いた。

「結果と考察」 1、3種類の組換え抗原を用いたELISA法のいずれも全虫体抗原を用いたELISA法により感度が優れていることが示された。2、組換えNcSAG1とNcSRS2を用いたELISA法では、非妊娠牛より流産牛の方が高い抗体価を有することが示された。ただし、組換えNcSAG1とNcSRS2を用いたELISA法では、流産牛と非流産牛を区別することはできなかった。このことから、NcSAG1とNcSRS2特異抗体は、妊娠期間中に生じた抗体である可能性が考えられた。3、流産牛のNcGRA7特異抗体は非流産牛より顕著に高かったことから、NcGRA7はネオスポラ原虫が引き起こす流産に関係する抗原である可能性が示唆された。そして、NcGRA7はネオスポラ原虫感染によって引き起こされる流産を検出するのに有力なマーカーになりうることが示唆された。

「結論」 組換えNcSAG1とNcSRS2を用いたELISA法では、流産牛と非流産牛の間に有意差が認められなかったのに対して、組換えNcGRA7を用いたELISA法では、流産牛と非流産牛の間に有意差が認められた。このことから、組換えNcGRA7を用いたELISA法は、ネオスポラ原虫感染による流産牛を検出できることが示唆された。