Identification of antigenic proteins from *Neospora caninum* and characterization of the surface antigen, NcSAG1

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ネオスポラ・カニナム由来抗原タンパク質の同定と表面抗原 NcSAG1 の解析

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List of abbreviations

BCA	- bicinochoninic acid
cDNA	- complementary deoxyribonucleic acid
CRISPIR /Cas9	- Clustered Regularly Interspaced Short Palindromic Repeats-Cas9
Ct	- cycle threshold value for quantitative PCR analysis
dpi	- days post infection
EDTA	-ethylenediaminetetraacetic acid
ELISA	- enzyme linked immunosorbent assay
EMEM	- Eagle's minimum essential medium
FBS	- fetal bovine serum
GRAs	- proteins secreted from dense granule organelles
GST	- glutathione S- transferase
HRP	- horseraddish peroxidase
iELISA	- indirect ELISA
IFAT	- immunofluorescence antibody test
IgG	- Immunoglobulin G
IHC	- immunohistochemical assay
IPTG	- isopropyl β-D-1-thiogalactopyranoside
kDa	- kilodalton unit
OD	- optical density
PBS	- phosphate-buffered saline
PBS-SM	- PBS containing 3% skimmed milk
PCR	- polymerase chain reaction
PV	- parasitophorus vacuoles
SDS-PAGE	- sodium dodecyl sulfate- polyacrylamide gel electrophoresis
Th1	- T helper 1 mediated (cellular) immunity
Th2	- T helper 2 mediated (humoral) immunity
Vero	- African green monkey kidney cells
WB	- western blot

General introduction

1. Historical background and prevalence rate of *Neospora caninum*

In Norway in 1984, a *Toxoplasma gondii*-like organism was identified as a parasitic causative agent causing neurological disorders in domestic dogs (Bjerkas et al., 1984). Subsequently, the parasite was identified as a new species and described as *Neospora caninum* in 1988 (Dubey et al., 1988a). Later, the parasite was isolated from naturally infected pups, propagated on cell culture and inoculated in mice and dog for production of specific antibodies (Dubey et al., 1988b). An immunohistochemical sassy was then developed to detect *N. caninum* in preserved tissue sections (Lindsay and Dubey, 1989). The first report regarding bovine neosporosis was in 1989 in a Holstein dairy herd with persistent abortions in New Mexico. *N. caninum*-tissue cysts were seen microscopically in sections of brain tissue of the aborted fetuses (Thilsted and Dubey, 1989). Then several reports related to bovine neosporosis as a cause of abortion in dairy and beef herds were recorded in different countries (Barr et al., 1990; Dubey et al., 1990a; Anderson et al., 1991; Barr et al., 1991a). Lindsay et al. (1999) identified the domestic dog as the final host of *N. caninum* parasite shedding the oocysts in its feces. Currently, bovine neosporosis is one of the most infectious abortifacient agents affecting the livestock industry worldwide.

A highly diverse seroprevalence rate of *N. caninum* antibodies has been reported in cattle in endemic countries. High prevalence rate has been reported in Turkey (60%), Beijing (43.4%), Pakistan (43.8%), while lower in some other countries including the United States (16%), United Kingdom (12%), Mexico (11.6%) (Dubey and Schares, 2011) and Egypt (18.9%) (Fereig et al., 2016). Although some evidence points to animal breeds as an important factor, the prevalence of *Neospora* infection in dairy cattle and beef has been closed in many countries including Argentina (80.9% and 73.0%) Brazil, (53.5% and 62.5%) and Spain (22.5% and 25.6%). The abortion associated with *N. caninum* parasite has been reported from most parts of the world based on pathological examination of the aborted fetus or various diagnostic techniques including detection of *Neospora* antigen by immunohistochemical assay (IHC), *Neospora* DNA by polymerase chain reaction (PCR) technique or antibodies identified in sera. The proportion of *N. caninum* associated abortions was around 77% in Mexico, 62 % in the USA, 57% in China, 40% in Switzerland, 34% in Brazil, 27% in Japan and 21% in Australia (Dubey, 2003a; Dubey and Schares, 2011). These results indicate the widespread of *Neospora* infection among cattle population. Although there is no firm evidence that *N. caninum* can induce clinical disease in humans (McCann et al., 2008), specific *Neospora* antibodies have been reported. In Brazil, *N. caninum* antibodies were detected in 38% of tested

human immunodeficiency virus-positive patients (Lobato, 2006). Other reports were obtained from the United States and Korea with prevalence of 6.7% (Nam et al., 1998; Tranas et al., 1999) and from Northern Ireland with prevalence of 8% (Graham et al., 1999). In Egypt, *N. caninum* prevalence rate in pregnant women was estimated at 7.9% (Ibrahim et al., 2009).

2. Morphological characterization and infective forms of *N. caninum* parasite

N. caninum is a coccidian parasite which is belonged to phylum Apicomplexa (Dubey et al., 2007). Some studies have suggested that *N. caninum* and *T. gondii* belonged to a common ancestor millions of years ago (Sibley, 2003). It is difficult to distinguish *N. caninum* and *T. gondii* tachyzoite under the light microscope. However, using the electron microscope, based on the appearance of rhoptries, is an easier task (Lindsay et al., 1993). *N. caninum* tachyzoites have a conoid with an apical polar ring, a three-layered plasmalemma, rod-shaped micronemes (n = up to 150), eight to eighteen rhoptries, a nucleus, mitochondrion, a Golgi complex, rough and smooth endoplasmic reticulum, micropores and dense granules (Speer and Dubey, 1989; Lindsay et al., 1993; Reid et al., 2012) (Fig. 1).

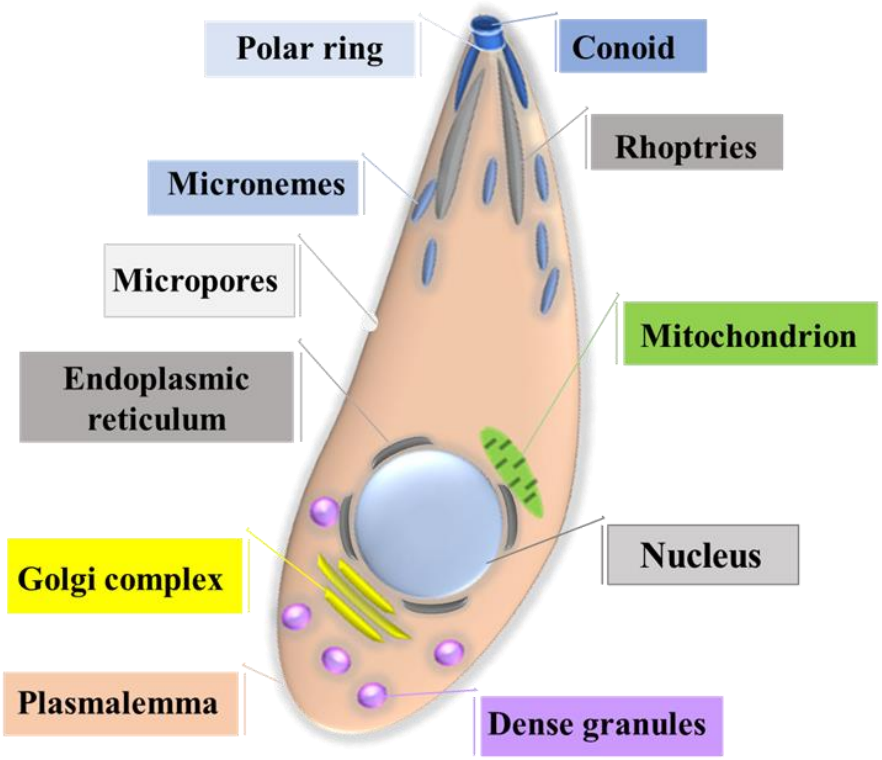


Fig. 1. Illustration for the ultra structure of the *Neospora caninum* tachyzoite showing the essential organelles (modified from Reid et al., 2012).

The tissue cysts are often round to oval in shape with a single thicker cyst wall in comparing to *T. gondii,* which protects the bradyzoites from the host's immune response. The *N. caninum* tissue cyst is observed mainly in muscle and the central nervous system (Dubey et al., 1988a; Dubey et al., 1990b; Dubey et al., 2005). Each tissue cyst contains approximately 100 bradyzoites (Silva and Machado, 2016). Bradyzoites are slender shape structure with resemble structure to the tachyzoites (Dubey and Lindsay, 1996), can survive at 4°C for more than 14 days (Speer et al., 1999).

The life cycle of *N. caninum* parasite involves three invasive stages. Environmental oocyst is the orally infectious that is sexually formed in epithelial cells of the small intestine of the definitive host. The sporulated oocysts of *N. caninum* are environmentally resistant and remain viable after various chemical and physical treatment (Alves Neto et al., 2011). Mature oocyst composes of two sporocysts, each of which contains four sporozoites that are released into the intestine of the host and transformed into tachyzoites (Dubey et al., 2004; Hemphill et al., 2006). Tachyzoite is the active stage of the parasite which has the ability to penetrate the host cells through 5 minutes of contact and multiplies asexually by a process termed endodyogeny (Dubey and Lindsay, 1996). Tachyzoites can invade different nucleotide types of host cells including mononuclear cells such as the leukocytes, through which the parasite disseminates to different organs (Hemphill et al., 2006). In this stage, the parasite replicates asexually inside an intracellular compartment formed from the host cell membrane (parasitophorous vacuole (PV)) (Buxton et al., 2002; Hemphill et al., 2006). Tachyzoites are released from the PV and invade neighboring cells, and clinical signs may develop at this stage. In response to the reaction of host immune system, the tachyzoites differentiate into slowly proliferating bradyzoites within tissue cysts (Goodswen et al., 2013). Bradyzoite is the quiescent stage of the parasite which persist the infection in the host long-life (Hemphill et al., 2006; Dubey and Schares, 2007). Bradyzoite- tachyzoite conversion usually takes place in the immunocompromised host.

3. Life cycle, host susceptibility and route of transmission of *N. caninum*

Although the morphological similarity between *N. caninum* and *T. gondii*, high differences have been recorded in the host range and clinical signs of such diseases. Generally, canines are the final host of *N. caninum*, while felines serve as definitive host of *T. gondii*. Cattle are more susceptible to neosporosis rather than sheep against toxoplasmosis. Virtually, *N. caninum* does not induce illness in humans, unlike *T. gondii* which has been reported as one of the most common parasites affecting humans causing

a wide variety of clinical signs ranging from influenza-like signs to serious disorders including abortion, congenital anomalies in pregnant women, and severe brain infection in immunocompromised persons. Finally, vertical transmission is the main route of infection of *N. caninum* rather than horizontal rout via oocysts (Dubey and Schares, 2007; Dubey, 2009).

Domestic dog and gray wolf are the only emphasized definitive host by demonstration of the viable oocysts in the faeces (Basso et al., 2009; Dubey et al., 2011). The coyote and Australian dingo are identified as final hosts for *N. caninum* experimentally by feeding tissues of *N. caninum*-infected calves (Gondim et al., 2004; King et al., 2010). Cattle, sheep, water buffaloes and white-tailed deer are proven as intermediate hosts to *N. caninum* by isolating the parasite from neonatally infected or adult asymptomatic animals (Dubey and Schares, 2007). *Neospora* DNA has been detected in some other species, such as goats, raccoon and rhinoceros. The serologic prevalence of specific *N. caninum*, antibodies has shown that many mammals including humans have been exposed to the parasite by accidental ingestion of the environmental oocyst (Dubey and Schares, 2007). Some of the putative dog preys including chicken (Mansourian et al., 2009), pigeons (Mineo et al., 2009), magpies and common buzzard (Darwich et al., 2012) have been shown to be intermediate hosts of *N. caninum* parasite.

The ingestion of tissue cyst is the most likely source of infection for dogs. Tissues of infected animal including brain, muscle, heart and liver are sources of infection (Cavalcante et al., 2011). Vertical transmission of *N. caninum* in dogs is induced experimentally (Cole et al., 1995a) and repeated transplacental infection of successive litters can occur (Dubey et al., 1990b). However, the results regarding transplacental transmission in dog were dramatically different. In a previous study, purebred labrador bitch gave birth of three *N. caninum* congenitally infected pups in her first litter. In the second litter of the same dam, all pups (n = 7) were apparently healthy, and six of them had no antibodies. Such results suggested that this mode of transmission cannot persist in the absence of the horizontal transmission (Dubey et al., 2005). Results of an experimental study suggest no role of the faecal transmission in the epidemiology of canine neosporosis (Bandini et al., 2011). Unlike dogs, vertical transmission of the parasite from the dam to its fetus is the main route for parasite transmission in cattle. Transplacental transmission has two different sources; exogenous through horizontal infection during pregnancy, or endogenous as a result of reactivation of persisted infection (Williams et al., 2009). Oral ingestion of the faecal oocyst is the only mode for the post-natal infection with the parasite (McCann et al., 2007). Some studies have detected *N. caninum* DNA in milk (Moskwa et al., 2007) and semen samples (Serrano-Martínez et al., 2007), suggesting sources of the

parasite transmission in cattle. However, venereal transmission is failed under experimental condition (Osoro et al., 2009) and there is no evidence regarding the lactogenic transmission of the parasite (Dubey et al., 2007). Transplacental transmission is induced in some other species including cat (Dubey and Lindsay, 1989), and mice (Cole et al., 1995b).

N. caninum is a parasite with heteroxenous life cycle where its development involves several host species (Dubey and Lindsay, 1996). Dog harbours both of the sexual and asexual stages of the parasite and acts as final and intermediate host, respectively (Lindsay et al., 1999; Gaitero et al., 2006). Infection usually occurs by swallowing contaminated tissue from infected animals or infected bovine placental tissue. The entire intra-epithelial cycle of the parasite in dog is still unknown (Dubey et al., 2004). Presumably, bradyzoites of the tissue cyst released in the intestinal epithelial cells of the final host, where the sexual development for the creation of oocyst takes place (Dubey, 1999). Unsporulated oocyst develops within 24-72 hours under different environmental conditions. Sporulated oocysts contaminate the food water and pasture of the intermediate host that is infected horizontally via ingestion of the faecal oocyst (Gondim et al., 2002). In the gastrointestinal tract of the intermediate host, sporozoites are released, transform into tachyzoites, disseminate to different organs including the placenta of pregnant animals (Hemphill et al., 2006). The parasite is able to cross into the placental villi and infect the fetus. The stage of gestation during infection determines the outcome of pregnancy. Infection in the early stage of pregnancy usually associates with abortion as a result of massive damage in the fetoplacental tissue (Gibney et al., 2008). Fetus absorption or stillbirth are also common at this stage. Infection in the later stage of pregnancy can result in the born of a survived fetus that may congenitally carry the infection. Congenitally infected heifer passes the infection to its progeny and persists the vertical transmission of *Neospora* parasite in the herd (Wouda et al., 1998). Recrudescence of the infection in the asymptomatic animal during pregnancy can occur as a result of host immunomodulation (Williams et al., 2009). Ingestion of the infected animal or placental tissue of infected aborted animal by the final host completed the life cycle of the parasite.

4. Clinical signs and economic impacts of *N. caninum* infection

Canine neosporosis is a fatal disease for dogs of all ages. However, most severe cases were observed in congenitally infected pups less than 6 months old (Dubey et al., 1988b). An infected adult dog exhibits various neurological signs including progressive cerebellar ataxia and cerebellar atrophy independent on the parasitized site (Garosi et al., 2010). Other neurological signs such as paresis, paralysis, seizures and head tilt may develop as complication of the central nervous system infection (Dubey and Lindsay,

1996; Garosi et al., 2010). Paralysis of the hind limbs is more common than the front limbs (Barber and Trees, 1996). Other disorders include paralysis of the jaw muscles, difficulty in swallowing, heart failure, muscle atrophy and flaccidity were reported (Hay et al., 1990; Odin and Dubey, 1993). The parasite can disseminate and infect different organs causing a variety of clinical signs including myositis, myocarditis, dermatitis, ocular lesions, hepatitis, pancreatitis and pneumonia leading to sudden death (Odin and Dubey, 1993; Barber and Trees, 1996; Barber et al., 1996). In puppies, the infection is mainly limited to inflammation of the muscles and nerve roots of the hind limbs and occasionally disseminates and develop encephalitis, myocarditis, hepatitis and pneumonia (Silva and Machado, 2016).

Reproductive failure is the only clinical sign observed in adult cattle infected with *N. caninum* (Dubey, 2003b). Virtually, abortion can take place from three months up to term of gestation while it is most often to occurs at five to six months of pregnancy. Probably, fetuses less than three months die in the uterus and absorbed (Dubey and Schares, 2011). Several reports regarding stormy abortion have been confirmed as neosporosis cases (Thornton et al., 1994; Yaeger et al., 1994) with high rate of productive failure up to 42.5% in some herds (Anderson et al., 1995). Both dairy and beef cattle are susceptible to the infection in any season of the year (Thurmond et al., 1995). Repeated abortion of the same animal is common in some herds. Nevertheless, a low rate of less than 5 % of repeated abortion cases was reported (Moen et al., 1995). Other clinical signs of infected pregnant cow including; fetus mummification or autolysis, stillborn, born a live clinically symptomatic or non-symptomatic fetus (Dubey and Schares, 2011). Neurological disorders are the main signs in *Neospora* infected calves. Flexion or hyperextension in the hind limbs and/or forelimbs are common. Neurological finding has been reported including ataxia decreased patellar reflexes, and loss of consciousness associated with myelitis, meningitis, and encephalitis (Parish et al., 1987). Affected congenital calves may have an asymmetric appearance of the eyes, exophthalmos (Bryan et al., 1994) and/or muscle degeneration which resulting in limb deformities (Dubey and de Labunta, 1993).

Bovine neosporosis is diseases that primarily affecting the reproductive system causing highly economic burden in cattle rising farms and associated industry in direct and indirect ways. The direct cost includes the cost of fetal loss, costs associated with culling, decrease in weight gain and milk production of an infected animal, while indirect costs are associated with replacement costs of the culled dam, time needs for rebreeding and expenses associated with diagnosis of aborted and infected case (Dubey, 2003a). The global economic impact of *N. caninum* livestock industry was calculated in ten different

countries, estimated at US $87.4 million in Argentine cattle industry, US $101.0 million per annum in the Brazilian beef industry, US $17.1 million annually in Canadian dairy industry, US $68.5 million and US $94.8 million per year in the Mexican dairy and beef industry, respectively. In Netherlands, neosporosis is estimated to cause a loss of US $700.00 at the level of dairy farms, while the annual economic burden of *Neospora* infection in the livestock industry was estimated at US $1.1 million in New Zealand beef farms, US $19.8 million in the Spanish dairy industry and US $546.3 million in US dairy cattle. The costs of *Neospora* abortion are estimated at an annual average of US $27 million in the United Kingdom (Reichel et al., 2013).

5. Control measurement and diagnostic approach of bovine neosporosis

Breaking the parasite infection cycle through prevention of consumption of infected tissue cyst by dogs by controlling rodents and the application of hygienic disposal of the placental tissue of aborted animal can assist control disease transmission (Dubey et al., 2007). Vaccine is the best disease control strategy as long as it protects against fetal loss, avoids vertical transmission. Despite promising results obtained with live tachyzoites vaccine or recombinant subunit vaccines against neosporosis (O'Handley et al., 2003; Williams et al., 2007; Nishimura et al., 2013), an effective and safe vaccine that prevents congenital infection of cattle with *N. caninum* is still unavailable in the market (Dubey et al., 2007). For the chemotherapeutic approach, toltrazuril and its derivative ponazuril has been found to be effective on *N. caninum* tachyzoites *in vitro* and *in vivo* in calves (Kritzner et al., 2002; Haerdi et al., 2006) and to control the transplacental transmission of *N. caninum* in experimentally infected pregnant mice (Gottstein et al., 2005). However, chemotherapy is believed to be an uneconomical approach for management of bovine neosporosis due to long term of treatment and withdrawal time that needs to get rid of drug residues in meat and milk. In addition, if treatment is available, it is difficult to determine the appropriate timing of treatment because of the high proportion of asymptomatic animals.

Currently, control measurement of *N. caninum* is based mainly on diagnostic approach. Diagnosis of bovine neosporosis can be achieved through the detection of *N. caninum* tachyzoites in infected lesions using IHC, detection of *Neospora* DNA by polymerase chain reaction (PCR) methodology in blood or semen samples, demonstration of specific antibodies in different types of samples using various serological tools, and assessment of the levels of proinflammatory cytokines as markers of infection (Ortega-Mora et al., 2006). For detection of specific antibodies, the immunofluorescence antibody test (IFAT) using fixed tachyzoites is the first applied technique to neosporosis (Dubey et al. 1988b) and it was used as a reference test for other techniques before (Björkman and

Uggla, 1999). Nevertheless, the test carried some disadvantage which limit its use. IFAT cut-off titer is varied between laboratories (1:100 to 1:640), making the standardization of IFAT results among different laboratories impossible. Additionally, the test requires a lot of training and experience, while result depends on personal assessment. Accordingly, interpretation of results is subjective with significant differences between laboratories (Ortega-Mora et al., 2006; Guido et al., 2016). Modified agglutination test (MAT) and Sabin-Feldman dye test were evaluated for the detection of *N. caninum* antibodies in different animal species (cattle, sheep, goats, pigs, rabbits, mice and rats) that showed good performance with specific reaction (Dubey et al., 1996).

The enzyme-linked immunosorbent assay (ELISA) is a suitable test for the detection of *Neospora* infection in large-scale surveillance studies. Different ELISAs have been developed including indirect ELISA (iELISA) and competitive-inhibition (CI)-ELISA. The iELISA using *N. caninum* crude extract antigens usually shows cross-reaction with *T. gondii* antibodies (Dubey and Lindsay, 1993), while the use of *N. caninum* purified antigens improved the specificity of the ELISA test significantly (Dubey and Schares, 2006). For this purpose, different recombinant antigens have been identified (Sinnott et al., 2017).

6. *Neospora caninum* diagnostic antigens

The molecules of *N. caninum* secretory organelles including rhoptries, micronemes and dense granules are essential for parasite attachment, invasion and successful establishment of infection within the host cell (Speer et al., 1999; Naguleswaran et al., 2002; Mercier 2005; Besteiro et al., 2011; Reid et al., 2012). Surface antigens of tissue-dwelling coccidia including *T. gondii*, *N. caninum* and *Sarcocystis* are thought to mediate attachment and invasion of the parasite to host cells, activating the host immunity and regulating the pathogenesis (Jung et al., 2004). Several serological tests are developed to detect antibodies to *N. caninum* based on *N. caninum* surface antigen1 (NcSAG1), including iELISA test (Chahan et al., 2003) latex agglutination test (LAT) (Moraveji et al., 2012) and rapid immunochromatographic test (ICT) (Liao et al., 2005a). The NcSAG1 shows high performance in the detection of *Neospora* specific antibodies in dog and cattle and has been reported as a marker for the activation of *N. caninum* (Chahan et al., 2003; Kubota et al., 2008; Hiasa et al., 2012a). NcSAG1-related sequence 2 (NcSRS2) is a potent diagnostic antigen which used extensively in the diagnosis of *N. caninum* infection. iELISA test based on NcSRS2 antigen shows high performance in the detection of *N. caninum* antibodies in bovine serum samples (Nishikawa et al., 2001; Borsuk et al., 2011; Dong et al., 2012; Pinheiro et al., 2013), milk samples of infected cattle (Schares et al., 2005) and serum samples of some other animal species including

dog and sheep (Nishikawa et al., 2001; Pinheiro et al., 2015). Blocking ELISA (b-ELISA) based on NcSRS2 was developed against bovine serum sample (Sinnott et al., 2015). NcSAG4 antigen is a bradyzoite stage-specific antigen, that correlated to chronic infection in cattle (Aguado-Martínez et al., 2008). Immunoblot (IB) method and iELISA test based on purified recombinant NcSAG4 antigen to detect antibodies against *N. caninum* in cattle were developed (Aguado-Martínez et al., 2008; Hu et al., 2011).

Dense granules molecules known as GRA proteins target the PV and establish successful infection (Vonlaufen et al., 2004). The *N. caninum* dense granule 2 (NcGRA2) is identified as a tachyzoite antigen and evaluated for the detection of *N. caninum* infection by ELISA test in dogs (Ellis et al., 2000; Jin et al., 2015). The diagnostic performance of NcGRA6 is evaluated by iELISA and LAT against bovine and canine neosporosis (Lally et al., 1996; Ghalmi et al., 2014) with good performance. The *N. caninum* dense granule 7 (NcGRA7) is expressed in tachyzoite and bradyzoite stages, while its specific antibodies are detected primarily in the early stage of infection and can be used as a suitable marker for parasite activation and developing signs. The recombinant antigen NcGRA7 exhibits high performance for detection of specific antibodies against *N. caninum* infection in different animal species based on the iELISA test (Aguado-Martinez et al., 2008; Hiasa et al., 2012a; Hiasa et al., 2012b). A previous study reports the utility of NcGRA7-based ELISA test as a serological tool for the detection of *N. caninum*-induced abortion in cattle (Huang et al., 2007), while a recent study unravels its immunoregulatory role in the pathogenesis of neosporosis (Nishikawa et al., 2018).

The family of microneme proteins (MIC) of all apicomplexan parasites is engaged in the formation of the moving junction and responsible for the gliding motility (Tomley et al., 2001; Alexander et al., 2005). Some of the *N. caninum* microneme proteins (NcMICs) are characterized and evaluated for its potential use as a diagnostic marker for neosporosis such as NcMIC10 (Yin et al., 2012), and NcMIC6 (Li et al., 2015). Some other diagnostic antigens are identified including cytoskeleton protein *N. caninum* profilin (NcPF) (Hiasa et al., 2012b), and serine protease NcSUB1 (Ybañez et al., 2013).

7. Aims of the study

Herein, this study investigated *N. caninum* for its elimination. At first, I focused on the development of serodiagnosis system based on recombinant antigens to improve the current diagnostic test for *N. caninum* infection. Additionally, I identified the virulence factors of *N. caninum* for better understanding the mechanism of infection, abortion and/or the vertical transmission of *N. caninum*. My research result will contribute on the development of vaccines and medications for control of *N. caninum* infection.

Currently, control measurement of *N. caninum* is relaying on accurate diagnosis for detection of infected animals. In this context, many diagnostic antigens have been identified. NcGRA7 is a potential diagnostic antigen against bovine and canine neosporosis. However, the protein is highly hydrophobic and completely insoluble (Lally et al., 1996). Accordingly, my aim in first chapter is to improve the hydrophilicity of NcGRA7 protein and investigate the antigenic properties using different fragments of the antigen.

Discrimination between *Neospora* abortion and *Neospora* infection is the basis of neosporosis management. In my second chapter, I worked on the establishment of a serological test for estimation of *Neospora* abortion. This was conducted through applying an antigenic comparison of numerous *N. caninum* antigens using aborted cattle sera of confirmed neosporosis cases.

Understanding the mechanism of *Neospora* abortion and the identification of the virulence factors is beneficial to provide a new goal for treatment or vaccine development. In the last chapter of this study, I aim to unravel the role of NcSAG1 gene as a virulence factor of *N. caninum* parasite and its possible role in the vertical transmission of the parasite through the generation of NcSAG1 knockout parasite using Clustered Regularly Interspaced Short Palindromic Repeats-Cas9 (CRISPR/Cas9) technique and using mouse model.

Chapter 1

Identification of the antigenic region of *Neospora caninum* dense granule protein 7 using ELISA

1-1. Introduction

N. caninum, an obligate intracellular protozoan parasite, can infect a wide range of domestic and wild animals, and also inflicts economic losses on the cattle industry (Dubey, 2003). *N. caninum* is widely distributed globally, and the infection rate is generally 10 to 40%, occasionally reaching 80% (Jenkins et al., 2002). The parasite is transmitted via the oral route and from the dam to the fetus in cattle and dogs (Dubey and Schares, 2001). Without any potent therapy or vaccine for both cattle and dogs, early and efficient diagnosis of *N. caninum* infection will assist the establishment of effective control policies against neosporosis (Dubey and Schares, 2001). High cost and cross reactions with *Toxoplasma gondii*, a related parasite, are the main disadvantages of most serodiagnostic tools (e.g., *N. caninum* commercial kits in dogs and equids) (Silva et al., 2007; Dubey and Desmonts, 1987). A recombinant protein-based, ELISA could overcome these drawbacks, and *N. caninum* surface antigens and dense granules (NcGRAs) have been reported to be promising tools because both are highly specific, sensitive and practical to use (Chahan et al., 2003; Huang et al., 2007).

With its specificity of antibody detection in infected animals and lack of cross reactivity against closely related *T. gondii*, NcGRA7 has strong potential for use as a diagnostic tool (Aguado et al., 2008; Hamidinejat et al.,2015). Two hydrophobic regions have been identified in NcGRA7 (amino acids 1–26, and 139–157). The first hydrophobic region appears to represent the signal sequence, while the other contains a putative transmembrane region. Consequently, NcGRA7 protein is highly hydrophobic and completely insoluble (Lally et al.,1996; Lally et al.,1997), and removing the signal peptide from the full-length gene greatly improved its expression in *Escherichia coli* (Hara et al., 2006). In this study, I mapped the antigenic regions of NcGRA7 by splitting the coding sequence into five fragments and examined the reactivity of each fragment against sera from *N. caninum* experimentally-infected mice, dogs and cattle. In addition, field sera of dog and cattle were tested. Identifying the relationship between antigenicity and animal species could potentially enable this antigen to be used diagnostically.

1-2. Materials and methods

Parasites and cell cultures

Nc-1 strain of *N. caninum* tachyzoites and PLK strain of *T. gondii* tachyzoites were propagated using monolayers of African green monkey kidney (Vero) cells in Eagle`s minimum essential medium (Sigma-Aldrich, St. Louis, MO) supplemented with 8% heat-inactivated fetal bovine serum. Tachyzoite purification was performed by washing the parasites and host-cell debris in cold PBS, and the final pellet was resuspended in cold PBS before passage through a 27-gauge needle and a 5.0-μm-pre-filter (Millipore, Bedford, MA, USA).

Animal serum samples

This study tested each NcGRA7 fragment against the serum samples obtained from the mice, cattle and dogs. For the mouse sera, female BALB/c mice (8 weeks old) were purchased from Clea Japan. *N. caninum* or *T. gondii* tachyzoites were intraperitoneally inoculated (1×10^5 and 1×10^3 tachyzoites respectively) into female BALB/c mice (9 weeks old, N=4). Serum (200 μl) was obtained from mice at 0- and 7-weeks post-infection. With the cattle, I evaluated the same samples used in a previous study (Hiasa et al., 2012a). Briefly, four serum samples collected from male Holstein calves aged 2–4 months at -13 and 29 days after intravenous infection with 1×10^7 tachyzoites of *N. caninum* Nc-1 strain were used. The reactivity of the sera from experimentally infected animals was confirmed by a commercial immunofluorescent antibody test slide (VMRD, Pullman, WA, USA) and ELISA based on recombinant NcSAG1 (Hiasa et al., 2012a; Hiasa et al., 2012b). Clinical serum samples from dogs (N = 19) were collected from animal hospitals located in Japan (Hiasa et al., 2012b). Cow serum samples (N = 20) were obtained from one Holstein dairy herd with a history of *Neospora*-associated abortions (Hiasa et al., 2012a). All serum samples were screened to detect *N. caninum* infection by NcSAG1-based ELISA (Hiasa et al., 2012a; Hiasa et al., 2012b). The protocol was approved by the Committee on the Ethics of Animal Experiments of the Obihiro University of Agriculture and Veterinary Medicine (Permit number 24-17, 25-66, 18-15).

Production and purification of recombinant proteins

The whole gene sequence of NcGRA7 (GenBank accession number, U82229.1) was sectioned into five fragments (Fig. 2A). Three of them (NcGRA7m, NcGRA7m3 and NcGRA7m4) were processed as recombinant proteins and two of them were synthetic peptides of NcGRA7m5 and NcGRA7m6 from Sigma Aldrich. The target sequences were PCR-amplified

from *N. caninum* Nc-1 complementary deoxyribonucleic nucleic acid (cDNA) using oligonucleotide primers that included a restriction enzyme sequence, a sense primer (5′-AC GAA TTC CGC TGG AGA CTT GGC A-3') for the three fragments, and the following anti-sense primers: NcGRA7m, 5′-GT GAA TTC CTA TTC GGT GTC TAC TTC CTG-3'; NcGRA7m3, 5′-GT CTC GAG TTA GAA TGT TCT CGC GAG ACC-3'; and NcGRA7m4, 5′-GT CTC GAG TTA ACG TTT TTT ACC GGG GAT-3') (Fig. 2A). *EcoRI* restriction enzyme sites were included in all the primers except the NcGRA7m3 and NcGRA7m4 anti-sense primers, which included *XhoI* sites. The digested PCR products were inserted into the pGEX-4T3 plasmid vector treated with the same restriction enzymes. The recombinant fragments were expressed as glutathione S-transferase (GST) fusion proteins in *Escherichia coli* BL21 (DE3), as described previously (Chahan et al., 2003) with slight modifications. The purities and quantities of the NcGRA7m-GST, NcGRA7m3-GST, and NcGRA7m4-GST recombinant proteins were assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), followed by Coomassie brilliant blue R250 staining (MP Biomedicals Inc., Illkirch-Graffenstaden, France). The three recombinant GST tagged proteins and GST itself were obtained with apparent molecular weights of 54 kDa, 38 kDa, 40.5 kDa and 27 kDa, respectively (Fig. 2B). Slight variations were observed in the apparent sizes of NcGRA7m3 and NcGRA7m4 than those expected of 40.7 kDa and 38 kDa, respectively. Because I confirmed that the NcGRA7m3 and NcGRA7m4 DNA sequences in the plasmids were consistent with those of the target regions, the composition ratio of hydrophilic to hydrophobic amino acids may have caused the unexpected gel shift. The protein concentrations were measured using a bicinchoninic acid protein assay kit (Thermo Fisher Scientific Inc., Rockford, IL, USA).

Indirect ELISA

ELISAs were performed as reported previously (Fereig et al., 2016) with slight modifications. Purified recombinant antigens or synthetic peptides (50 µl aliquots), each at a final concentration of 0.1 µM in carbonate-bicarbonate buffer (pH 9.6), were coated onto the ELISA plates (Nunc, Roskilde, Denmark) at 4°C overnight. The plates were washed once with PBS containing 0.05% Tween 20 (PBS-T) and blocked with PBS containing 3% skimmed milk (PBS-SM) for 1 h at 37°C. The plates were washed once with PBS-T, and 50 µl aliquots of the serum samples, diluted 1:600 for mouse and dog sera or 1:300 for cattle sera in PBS-SM, were added to the wells. The plates were incubated at 37°C for 1 h. After washing six times with PBS-T, the plates were incubated with horseradish-peroxidase (HRP)-conjugated IgG (Bethyl Laboratories, Montgomery, TX, USA) at 37°C for 1 h. HRP-conjugated goat anti-mouse IgG1 and IgG2a diluted 1:15,000 with PBS-SM, sheep anti-bovine IgG1 and IgG2 diluted 1:10,000 with PBS-SM, and

goat-anti dog IgG1 and IgG2 diluted 1:15,000 with PBS-SM were used as the secondary mouse, cattle and dog sera, respectively. The plates were washed an additional six times before the substrate solution [0.1 M citric acid, 0.2 M sodium phosphate, 0.003% H2O2 and 0.3 mg/ml 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (Sigma-Aldrich) was added to each well in 100 µl aliquots. The absorbance at 415 nm after 1 h of incubation at room temperature (RT) was measured using an ELISA reader. The readings for the recombinant antigens were subtracted from those of the GST protein.

1-3. Results

Using an IgG1 and IgG2a-based ELISA with the mouse sera, the three NcGRA7m, NcGRA7m3 and NcGRA7m4 fragments could differentiate *N. caninum*-infected mouse sera from uninfected mouse sera, and no cross-reactivity against the *T. gondii*-infected mouse sera was observed (Fig. 3A). The reactivity of the three fragments against the *N. caninum*-specific mouse IgG1 and IgG2a did not differ. Furthermore, for the experimentally infected dog sera, compared with the negative control sera from them, the three fragments each reacted strongly against IgG2 in particular, and a statistically significant difference was observed between NcGRA7m and NcGRA7m3 (Fig. 3B). The IgG1 in the sera from the *N. caninum*-infected dogs reacted against the three fragments also, but with lower reactivity than of IgG2. The IgG1 and IgG2 antibody responses indicate the presence of Th2 and Th1-immune responses in the dogs, respectively (Stevens et al., 1988). Accordingly, my results suggest that anti-NcGRA7 IgG2 may be an indicator of the cellular immune response against *N. caninum* infection in dogs. In dog field sera, the three fragments (NcGRA7m, NcGRA7m3 and NcGRA7m4) showed low reactivity against the IgG1 antibodies compared with the reactivity of the IgG2 antibodies, indicating the similar results of the experimentally infected sera. In comparison with the reaction of IgG2 antibodies in the experimentally infected dog sera, the levels of IgG2 antibodies against NcGRA7m3 were higher than those against NcGRA7m and NcGRA7m4. These results suggest that the antigen structure of NcGRA7m3 is critical for the recognition of *N. caninum*-specific IgG2 antibodies (Fig. 4A). Additionally, these results revealed the high diagnostic capabilities of the three fragments (NcGRA7m, NcGRA7m3 and NcGRA7m4) for *N. caninum* infections in mice and dogs, and only the C-terminal regions of the synthetic peptides (NcGRA7m5 and NcGRA7m6) were unreactive (Fig. 3). The NcGRA7 fragment (amino acids 27 to 160) has potential, therefore, as a diagnostic tool for *N. caninum* infection in dogs.

Conversely, when the different fragments were tested against the cattle sera, high IgG1 levels were observed for the NcGRA7m fragment compared with NcGRA7m3, NcGRA7m4,

NcGRA7m5 and NcGRA7m6 (Fig. 3C). No IgG2 reactivity against any fragment was detected, which is consistent with the Hiasa et al. study results (2012a). In cattle field sera, IgG1 antibodies against NcGRA7 were predominant compared with the IgG2 antibodies. The levels of IgG1 antibodies against NcGRA7m were significantly higher than those of IgG1 antibodies against NcGRA7m3 or NcGRA7m4. These results were consisted with the results of the experimentally infected sera (Fig. 4B). Thus, when NcGRA7 undergoes fragmentation in cattle this can negatively affect its antigenic potential. This result suggests that the structure of the mature NcGRA7 without the signal peptide may be essential for IgG1-specific antibody detection, and the result is consistent with the observations of numerous reports that have indicated that NcGRA7 predominantly enhances humoral immune responses as indicated by high IgG1 production (Hiasa et al., 2012a; Nishikawa et al., 2007).

1-4. Discussions

The results of the current study revealed antibody production differences against NcGRA7 among mouse, dog and cattle, suggesting a species-specific immune response against *N. caninum* infection in the different host animals. Distinct variation in the humoral mechanisms against *N. caninum* infection has also been reported for different cattle breeds (Santolaria et al., 2011). This variation in antibody production may be attributed to functional differences in the immunoglobulin subclasses among the host species (Haley, 2017). Furthermore, genetic diversity in the IgG and cell-mediated effector molecule repertoires can determine the types and levels of the immune responses triggered, including antibody production (Cooper and Alder, 2006). In particular, the enormous variation in the genetic makeup of the major histocompatibility complex not only among different animal species but also within a species, are considered to underlie the species-specific immune response (Kelley and Walter, 2005).

1-5. Summary

NcGRA7 is a potent diagnostic antigen of *N. caninum*. Some studies have reported on the difficult expression, low yield, and variable degree of solubility of recombinant NcGRA7. Here I aimed to unravel the possible causes for these issues and tested NcGRA7 antigenicity in ELISAs. The NcGRA7 coding sequence (217 amino acids) was split into five amino acid regions: NcGRA7m, m3, m4, m5 and m6. The five aforementioned fragments were evaluated using *Neospora* experimentally infected sera collected from mice, cattle and dogs. Three fragments, NcGRA7m, NcGRA7m3 and NcGRA7m4, exhibited high antigenic properties when tested against experimentally-infected mice and dog sera by ELISA. The C-terminal region (NcGRA7m5

and NcGRA7m6) were unreactive. In clinically infected dog sera, NcGRA7m3 fragment exhibited significant high antibody levels in comparison to other diagnostic fragments (NcGRA7m and NcGRA7m4). While NcGRA7m fragment was the only antigenic region against experimentally and naturally infected cattle sera. Accordingly, this study reported the full length of NcGRA7 antigen lacking the transmembrane region (NcGRA7m) as the highly antigenic region in the detection of *Neospora* infection in different animal species. The current study presents valuable information about the antigenic regions of NcGRA7. The varied performance of NcGRA7 with cattle and dog sera highlights the need to consider the species factor when developing a diagnostic tool based on this antigen.

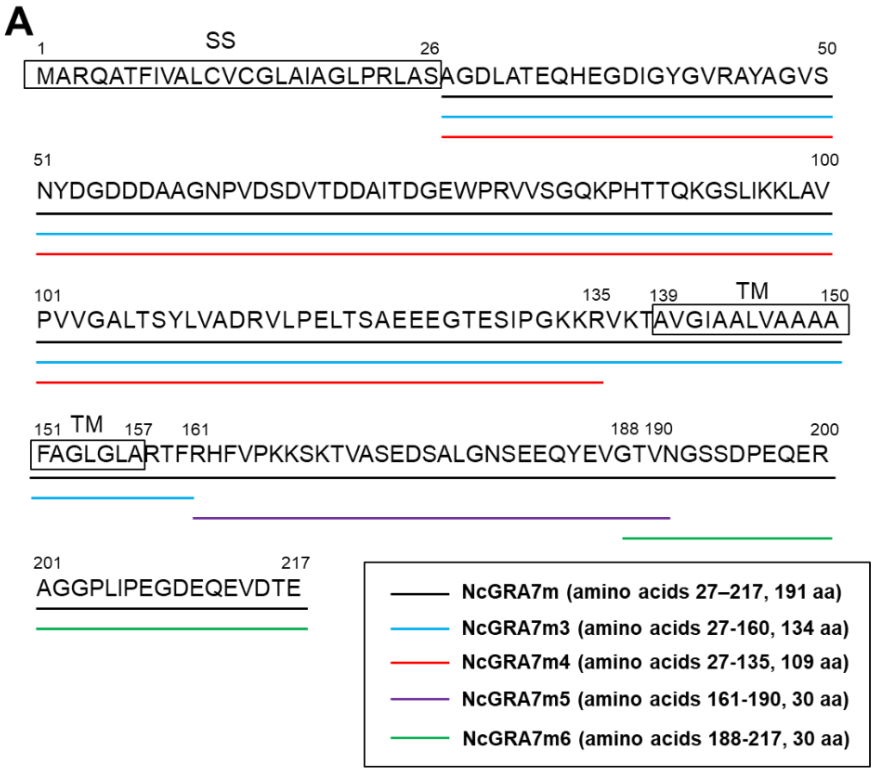


Fig. 2. (A) Schematic depiction of the NcGRA7 fragments used in this study. The amino acid lengths of the fragment were 191, 134, 109, 30 and 30 for NcGRA7m, NcGRA7m3, NcGRA7m4, NcGRA7m5 and NcGRA7m6, respectively. SS, signal sequence; TM, transmembrane domain. In NcGRA7m fragment (amino acids 27–217), the first 26 amino acids (amino acids 1–26) which represent the signal peptides were removed. In NcGRA7m4 fragment (amino acids 27–135), the transmembrane region (amino acids 139–157) was removed. The C-terminal region (amino acids 161–217) was split in NcGRA7m3 (amino acids 27–160). The C-terminal region was dissected into two different regions; NcGRA7m5 (amino acids 161–190) and NcGRA7M6 (amino acids 188–217). (B) SDS-PAGE analysis of purified recombinant NcGRA7m-GST (lane 1), NcGRA7m3-GST (lane 2), NcGRA7m4-GST (lane 3), and GST (lane 4). All the recombinant proteins were GST fusions. M, molecular mass markers.

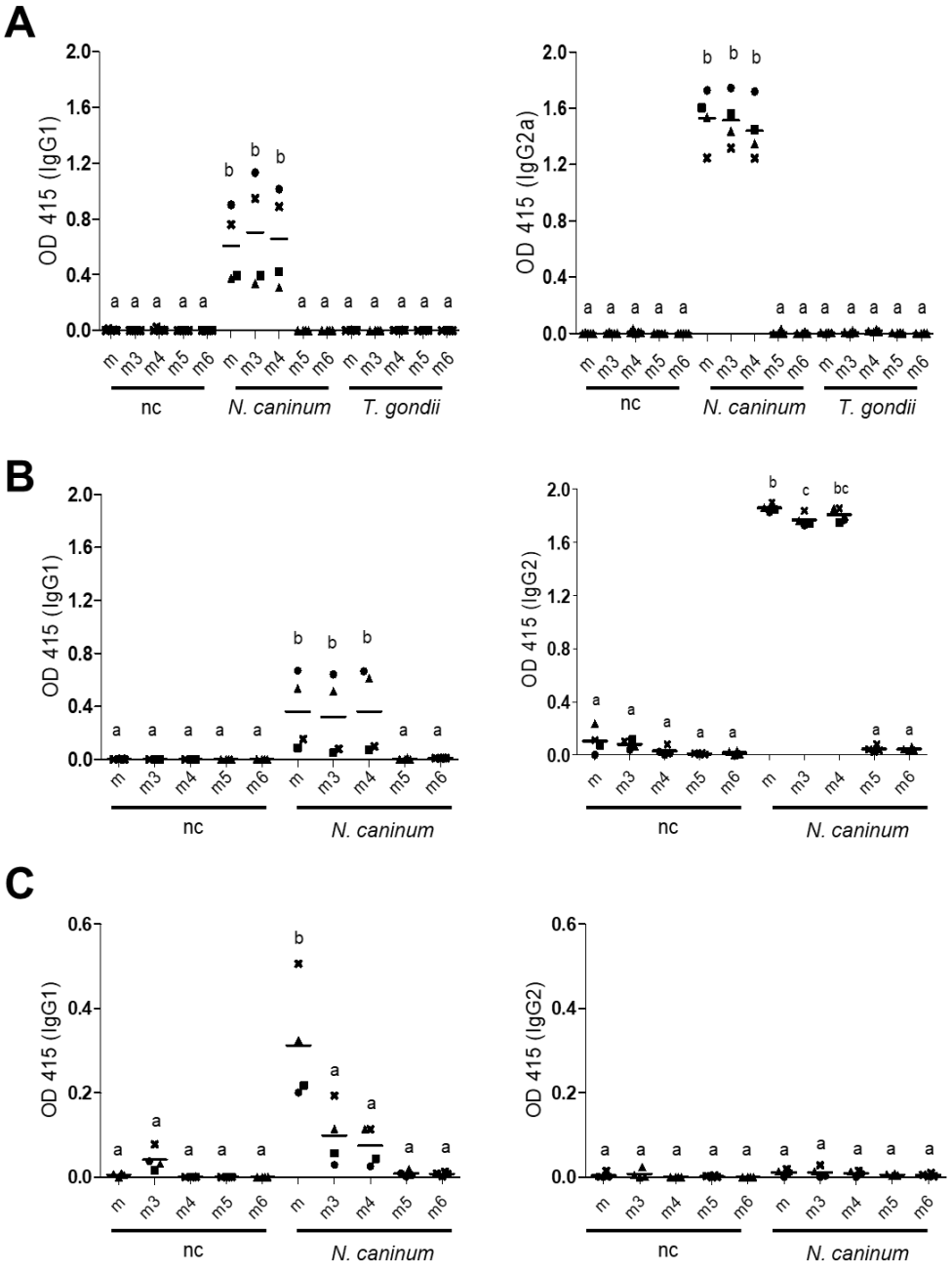


Fig. 3. **Reaction of sera from experimentally infected animals.** (A) Reaction of mouse IgG1 and IgG2a antibodies against the NcGRA7 fragments. (B) Reaction of dog IgG1 and IgG2 antibodies against the NcGRA7 fragments. (C) Reaction of cattle IgG1 and IgG2 antibodies against the NcGRA7 fragments. The data represent two independent experiments with similar results. The different letters above the bars in the graphs indicate statistically significant differences among the groups tested (one-way ANOVA plus Tukey–Kramer post hoc analysis, $P < 0.05$). m, NcGRA7m-GST. m3, NcGRA7m3-GST. m4, NcGRA7m4-GST. m5, NcGRA7m5. m6, NcGRA7m6. nc, sera from uninfected animals. Triangle, sample no. 1; circle, sample no. 2; ×, sample no. 3; square, sample no. 4.

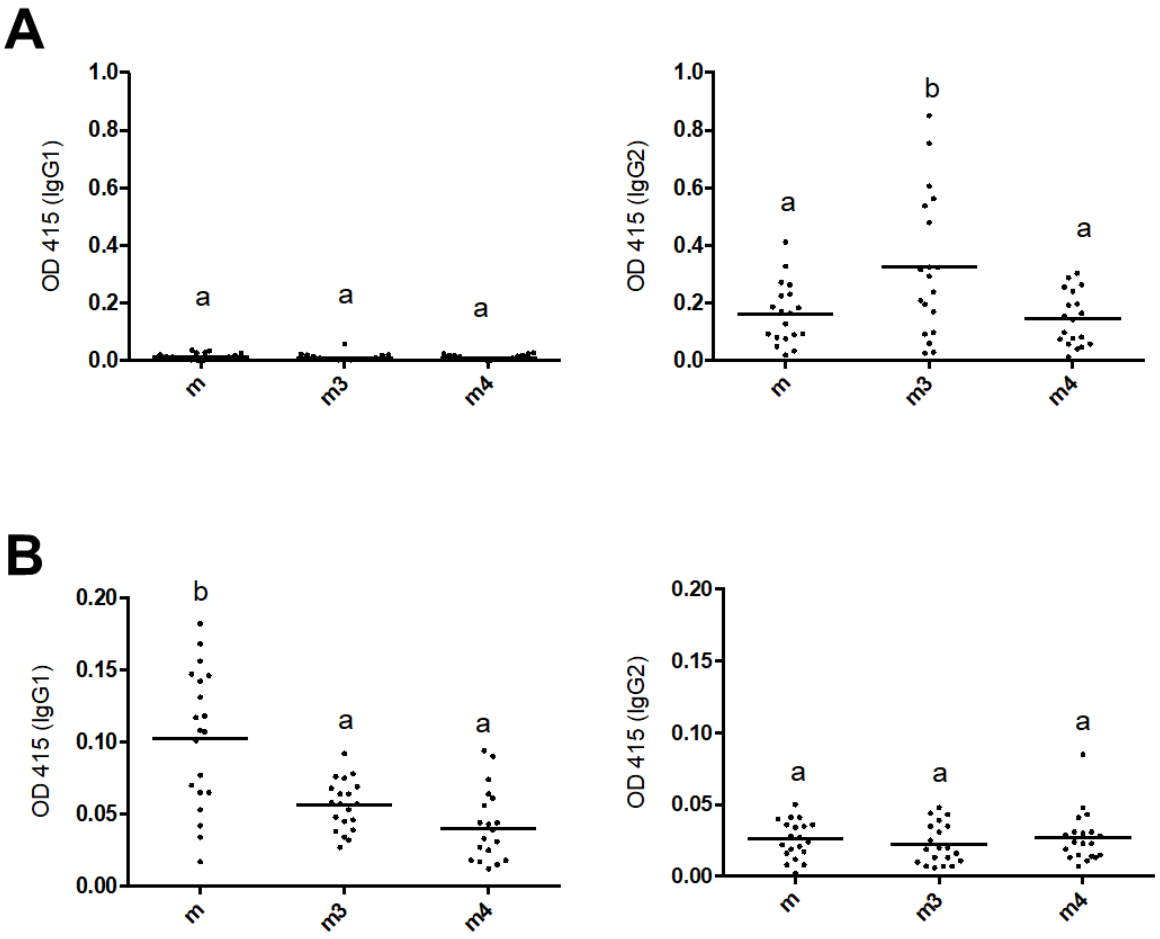


Fig. 4. Reaction of field sera. (A) Reaction of dog IgG1 and IgG2 antibodies against the NcGRA7 fragments. (B) Reaction of cattle IgG1 and IgG2 antibodies against the NcGRA7 fragments. The data represent two independent experiments with similar results. The different letters above the bars in the graphs indicate statistically significant differences among the groups tested (one-way ANOVA plus Tukey–Kramer post hoc analysis, $P < 0.05$). m, NcGRA7m-GST. m3, NcGRA7m3-GST. m4, NcGRA7m4-GST.

Chapter 2

Evaluation of *Neospora caninum* serodiagnostic antigens for bovine neosporosis

2-1. Introduction

N. 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. In addition to neurological disorders, inability to rise, and below average birth weight can be noticed in newborn calves. Unlike the definitive host, vertical transmission of the parasite from infected dams to their progeny is the main route of infection (Dubey, 2003). Congenitally infected calves can pass the infection onto their progeny and perpetuate the vertical transmission cycle of the parasite in herds (Anderson et al., 1997). By contrast, horizontal infection via ingestion of contaminated food or water containing fecal oocysts from infected dogs is primary route of epidemic transmission (McAllister et al., 2000; Jenkins et al., 2002). 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 (Reichel et al., 2013).

Despite extensive efforts to develop effective vaccines or pharmacological treatments for neosporosis (Nishikawa, 2017), 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 IHC is a routine diagnostic test for detecting *N. caninum* antigens in infected tissue (Lindsay and Dubey,1989; Dubey and Schares, 2006). However, low sensitivity of IHC, especially in autolyzed tissue, has been reported (De Meerschman et al., 2005).

Detection of *N. caninum* antibodies can be achieved through many serological tests including IFAT and iELISA assays. 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 (Guido et al., 2016). 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 (Sinnott et al., 2017).

NcSAG1 is an immunodominant antigen expressed in tachyzoites and downregulated during the tachyzoite-to-bradyzoite conversion (Vonlaufen et al., 2002). Recombinant NcSAG1 based-iELISA is an effective serodiagnostic tool for detection of *N. caninum* infection in cattle and dogs (Chahan et al., 2003; Kubota et al., 2008). 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 (Aguado et al., 2008; Hiasa et al., 2012a; Hiasa et al., 2012b). The role of NcGRA7 in regulation of *N. caninum* pathogenesis through modulation of host immune responses was recently clarified (Nishikawa et al., 2018). The diagnostic performance of NcGRA6 was reported for cattle sera (Lally et al., 1996). The rNcGRA6 protein is an efficient immunomodulator and potential vaccine candidate for *N. caninum* infection in mouse models (Fereig et al., 2019a). *N. caninum* cyclophilin antigen (NcCyP) is a secretory protein which triggers production of host interferon-gamma (IFN-ɣ), contributes to host cell migration (Tuo et al., 2005; Kameyama et al., 2012), and induces potent protection against *N. caninum* infection in mice in a Toll-like receptor 2-dependent manner (Fereig et al., 2019b). Dense granule protein 14 (NcGRA14) is a recently-described *N. caninum* protein and no reports have examined its antigenic performance (Liu et al., 2013).

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 (Huang et al., 2007; Aguado et al., 2008). Accordingly, I 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.

2-2. Materials and Methods

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.

Parasites and host cell cultures

The *N. caninum* (Nc-1) strain was propagated in Vero cells cultured in Eagle's minimum essential medium (EMEM; 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 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).

Preparation of recombinant antigens

This study used five *N. caninum* recombinant antigens: NcSAG1, NcGRA6, NcGRA7, 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 1) 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 *E. coli* BL21 (DE3) cells as 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 a sonication buffer (50 mM Tris-HCl, pH 8, 50 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA) and 1 mM dithiothreitol) and 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 RT (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 (Fig. 5).

Sera from experimentally-infected animals

Antigens (NcSAG1, NcGRA6, NcGRA7, NcGRA14, and NcCyP) 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 intravenously inoculated 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 (Abdelbaky et al., 2018).

Field serum samples

The antigenic properties of highly diagnostic antigens (NcSAG1, NcGRA6 and NcGRA7) 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. 9): (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*. I 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.

iELISA

iELISAs were performed as reported previously (Abdelbaky et al., 2018) 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 a 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% H2O2, 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 RT 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, I 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.

Statistical analysis

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

2-3. Results

Assessment of recombinant antigens using sera from experimentally-infected animals

First, the diagnostic performance of antigens such as NcSAG1, NcGRA6, NcGRA7, NcGRA14, and NcCyP and 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. 6A). 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. 6B). This result reflects 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. 6). These results indicated the high efficacy of NcSAG1, NcGRA7 and NcGRA6 recombinant antigens for detection of *N. caninum* infection in mice and cattle.

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. I used the maternal sera to

validate the performance of three highly diagnostic *Neospora* antigens (NcSAG1, NcGRA6 and NcGRA7) using iELISA, and compared these results against cELISA as a reference test. The three aforementioned antigens were differentially recognized in IgG-based iELISAs (Fig. 7). 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 (Table 2). 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 (Table 2). 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. 8). 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 (Table 2).

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. 9). These results indicated the usefulness of both antigens as serological tools for estimation of *Neospora* abortion in cattle during abortion outbreaks.

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. 10). 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.

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. 11). My results indicated that high levels of anti-NcSAG1 and anti-NcGRA7 antibodies were specifically associated with *Neospora* abortion rather than neurological symptoms.

2-4. 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 (Dubey and Schares, 2006). 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 (IHC) of *N. caninum* antigens (Dubey and Schares, 2006). However, IHC is an invasive and postmortem test with limited sensitivity (De Meerschman et al., 2005), and often shows marked cross-reactivity against *T. gondii* (McAllister et al., 1996a). Additionally, the test is laborious and expensive. ELISA against recombinant antigens for detection of specific antibodies is a simple and rapid test requiring a 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 (Sinnott et al., 2016). However, identifying appropriate cut-offs is essential for proper design of serological assays. *Neospora*-infected animals usually show lower levels of antibodies as compared with aborted cases (Dubey et al., 1997). Thus, for identification of infected cattle, serological tests with higher sensitivity and lower cut-offs are required (Jenkins et al., 2002; Schares et al., 1999; Alvarez et al., 2003). Recombinant antigen-based ELISAs against NcSAG1 and NcGRA7 are potential serological tools for detection of *N. caninum* infection in cattle (Chahan et al., 2003; Hiasa et al., 2012a), and dogs (Kubota et al., 2008; Hiasa et al., 2012b). 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, NcGRA6 and NcGRA7 antigens were selected for subsequent investigations using sera from *Neospora*-aborted cows.

The three antigens (NcSAG1, NcGRA6, and NcGRA7) 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 as 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 (Alvarez et al., 2013).

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 (McAllister et al.,

1996b; Dubey et al., 1997; Quintanilla et al., 2000). 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. A previous study conducted in our lab suggested the possibility of using serological testing for diagnosis of neosporosis as a cause of abortion among cattle (Huang et al., 2007). A previous study in our lab, 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 (Huang et al., 2007). 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 (Innes et al., 2002). My 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. 10A, B and C) showed marked and sudden formation of specific antibodies against NcSAG1, particularly in *Neospora*-seropositive animals (Fig. 10B and C). The detection of antibody level against both antigens in the seronegative group during pregnancy (Fig. 10A) 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 (Dubey et al., 2007).

Up to 95% of live-born calves from *Neospora*-seropositive dams can be congenitally infected and clinically normal (Pare etal., 1996). However, clinical signs, including neurologic signs, have been reported in calves less than 4 months of age (Barr et al., 1991b). Thus, I investigated the specific reactivity against NcSAG1 and NcGRA7 of serum samples from neurologically and non-neurologically symptomatic calves

with *N. caninum*. My 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. My 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.

2-5. Summary

Recently, significant advances have been made in serodiagnosis of *N. caninum* via specific antibody detection against antigens. In the current study, I 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, my 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.

Table 1. 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
	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-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., 2019a
	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
	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
	2-reverse	5’-GC GAA TTC TTA CAA CAA ACC AAT GTC CGT-3’	*Eco*RI		

Table 2. *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 tissue from the aborted dams were tested for *N. caninum* antigens using an immunohistochemical (IHC) assay used for diagnosis of neosporosis.

Fig 5. 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.

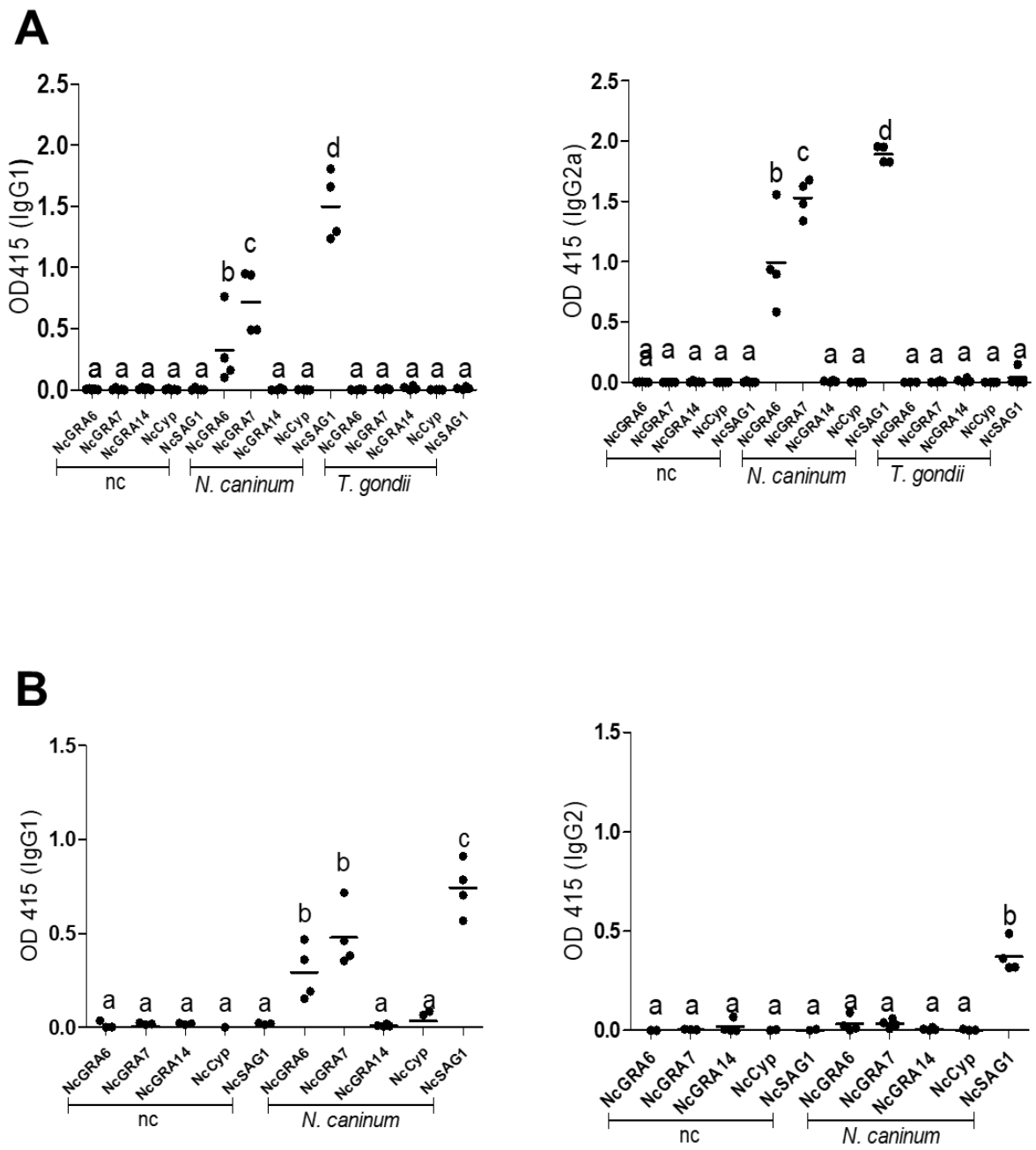


Fig. 6. **Evaluation of *N. caninum*-derived recombinant antigens using sera from experimentally-infected animals.** Antibody levels of IgG1 and IgG2a in sera of experimentally-infected mice (A) and antibody levels of IgG1 and IgG2 in sera of experimentally-infected cattle (B) were assessed against different *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, $P < 0.05$).

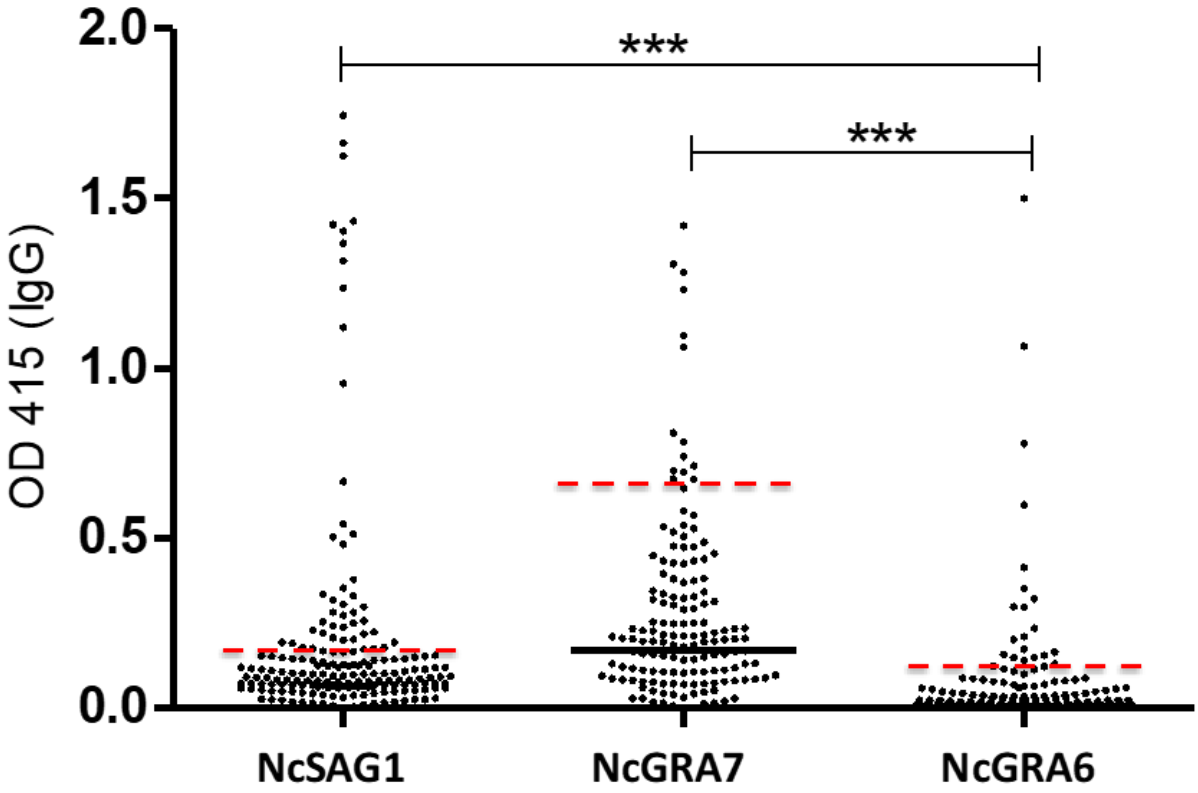


Fig 7. Validation of recombinant antigen-based iELISA using sera from aborting cattle. Comparison of *N. caninum* antigens (NcSAG1, NcGRA7 and NcGRA6) for analysis of aborted cattle sera (n = 164) using IgG-based iELISA. Higher prevalence rates were recorded against NcSAG1 (28.0%) followed by NcGRA7 (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 ($P < 0.0001$).

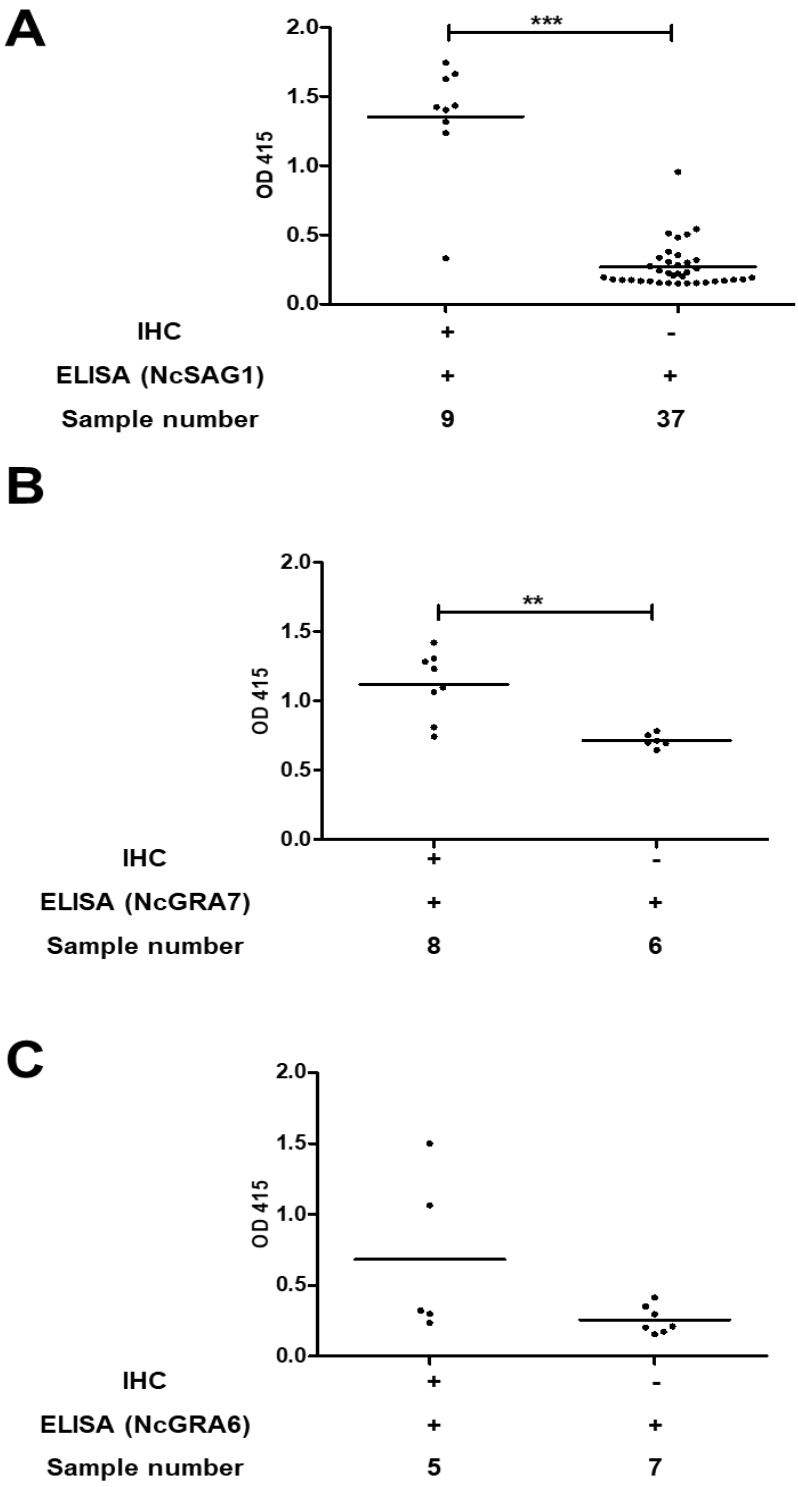
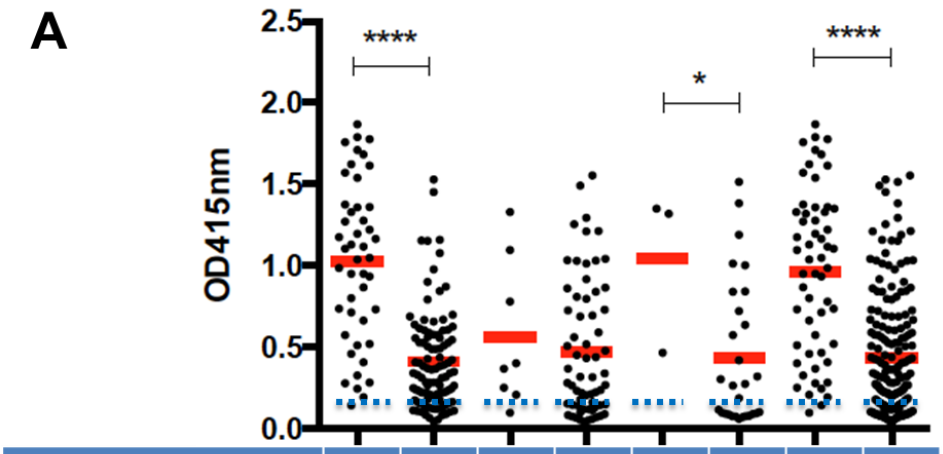
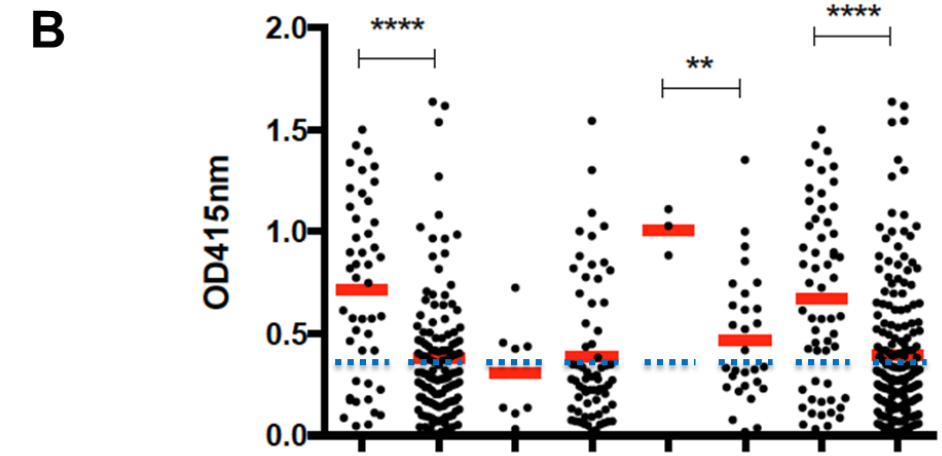


Fig. 8: Determination of antibody levels of IHC-positive samples and iELISA-positive samples using sera of aborted cattle. Comparison of antibody levels of IHC positive (+) and negative (-) in *Neospora*-positive samples determined using each iELISA. A: NcSAG1, B: NcGRA7, C: NcGRA6. Solid lines indicate average values. The significance of differences was analyzed using the Mann-Whitney *U* test because the data were non-normally distributed (*: $P < 0.05$, **: $P < 0.01$, ****: $P < 0.0001$).

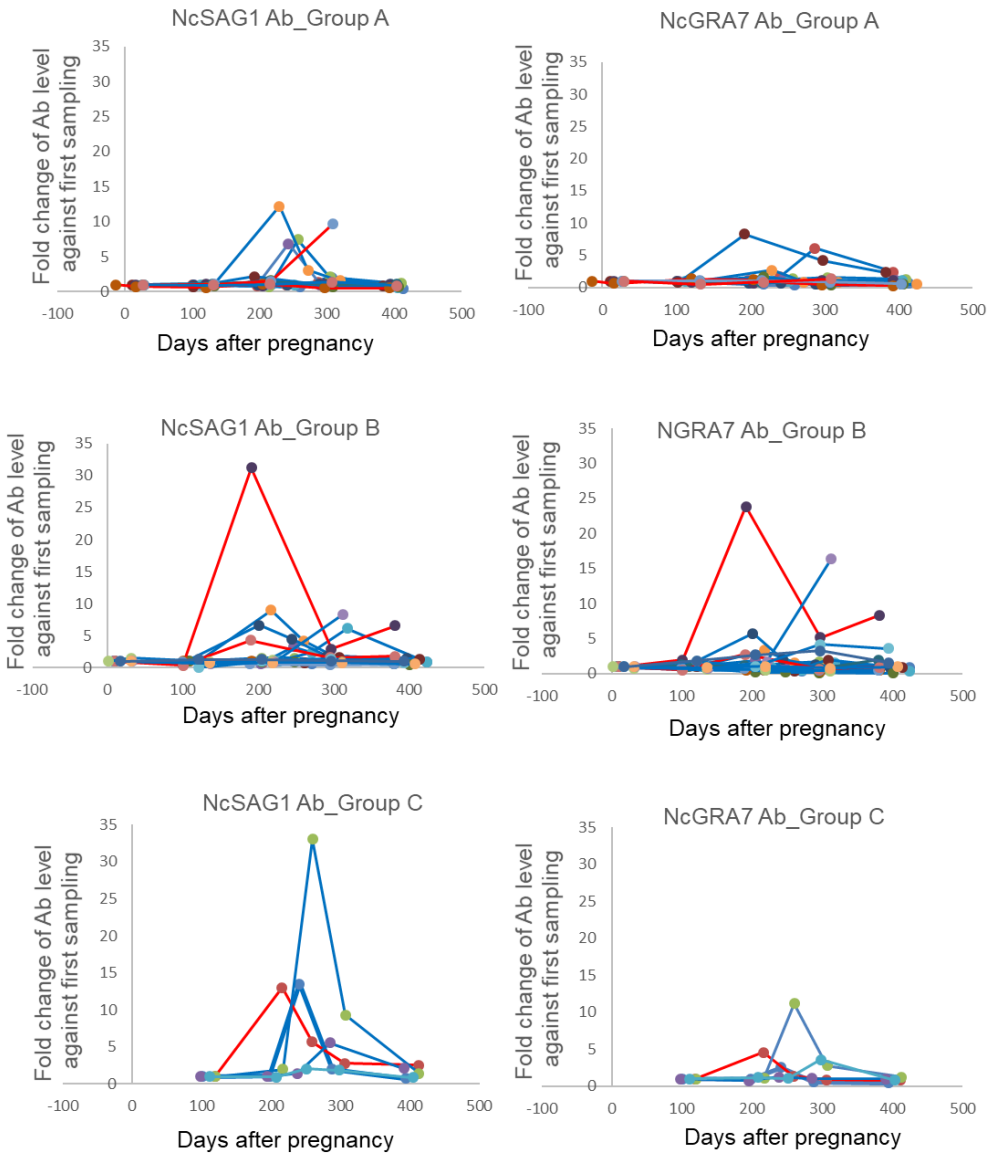


Abortion	+	-	+	-	+	-	+	-
Sample number	46	125	8	66	3	29	57	220
Seropositive rates	97.8%	85.6%	87.5%	68.2%	100%	84.2%	96.5%	76.3%
Category	Lactation		Dry		First pregnancy		Total	



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	

Fig. 9: 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.



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

Fig. 10: Dynamics of anti-NcSAG1 and anti- NcGRA7 antibody levels in pregnant cattle. Data from 36 sera collected from pregnant dams with different serological status to *N. caninum* and different histories of abortion are shown. Lines with different colors indicate different animals. Red lines indicate cattle that experienced abortion or stillbirth during the sampling period. Animals were classified into Groups A, B and C according to the table. (a): Abortion was confirmed before the time of first sampling, (b): *Neospora*-specific antibodies were detected at the first sampling by iELISA using either NcSAG1 or NcGRA7. The fold change was calculated by dividing the optical density (OD) value of each sampling point against initial sampling point for the relevant animal tested by NcSAG1 or NcGRA7-based ELISA.

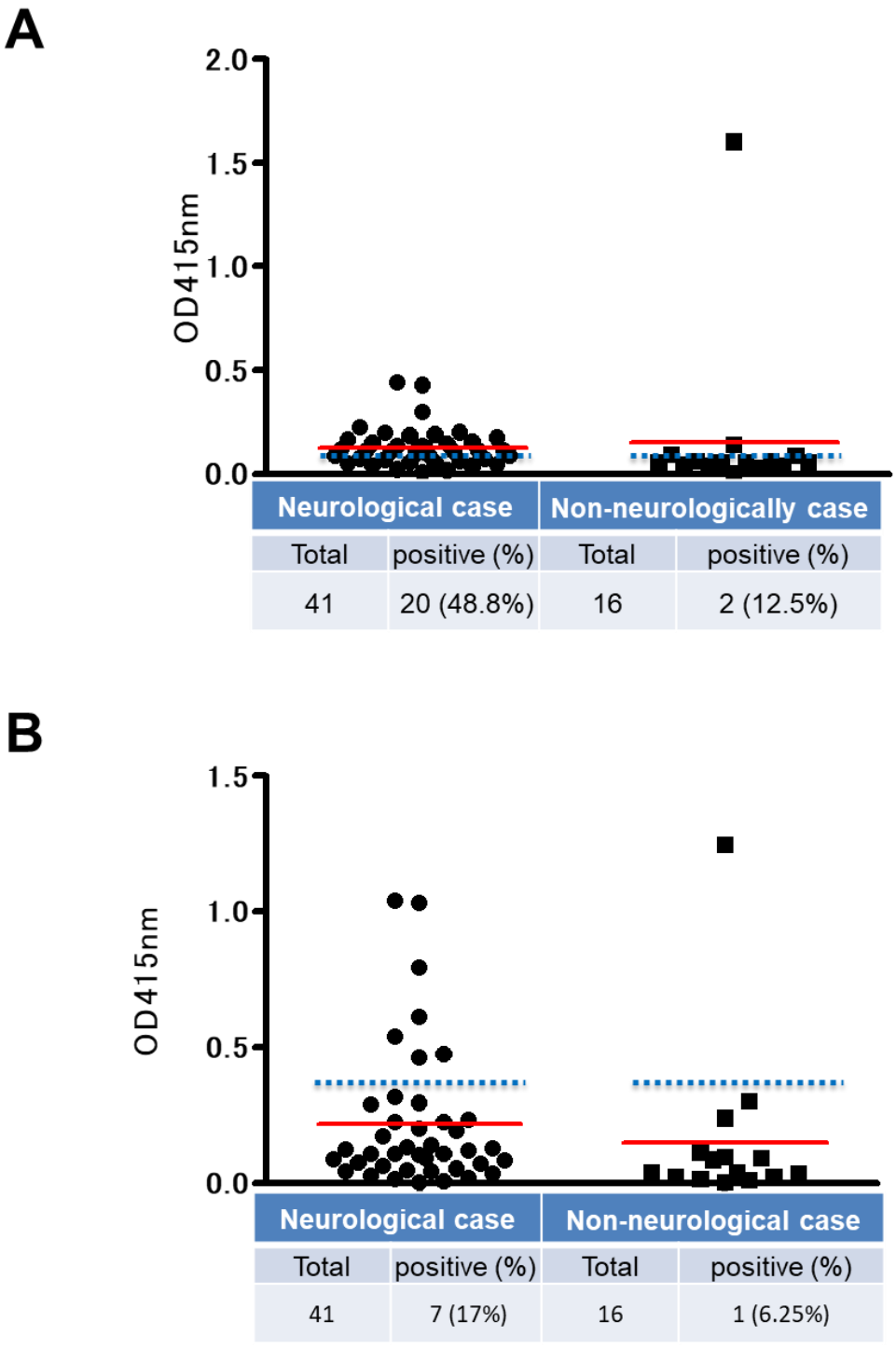


Fig. 11: **Levels of anti-NcSAG1 (A) and anti-NcGRA7 (B) antibodies in neurologically symptomatic calves.** Results of iELISAs using sera from neurologically symptomatic (n = 41) and non-symptomatic calves (n = 16) are shown. No significant differences were observed between the two groups. Non-normally distributed variables (A) were compared using the Mann-Whitney *U* test and normally-distributed variables (B) were compared using the Student's *t* test. Solid red lines indicate the average values of samples. Dotted blue lines indicate the cutoff value.

Chapter 3

Neospora caninum surface antigen 1 is a major determinant for pathogenesis of neosporosis in non-pregnant and pregnant mice

3-1. Introduction

Neospora caninum, which is a closely related protozoan parasite to *Toxoplasma gondii*, can infect a wide range of intermediate hosts. *In vitro*, *N. caninum* has the capacity to be maintained in a wide range of cell cultures. However, in contrast to *T. gondii*, *N. caninum* is a primary causative agent of abortion or foetal abnormalities in cattle, and to a lesser extent, in sheep and other ruminants (Goodswen et al., 2013).

Three developmental and infective stages have been reported to *N. caninum*; tachyzoite (rapidly growing stage), and bradyzoite (slowly growing stage) and sporozoite (exist in oocyst). The sexual cycle of *N. caninum* occurs in dogs and some other canids which act as definitive hosts that are affected by neurological clinical, while many other animals including cattle act as intermediate hosts that harbor only asexual stage (Dubey and Scahres, 2011; Fereig et al., 2020).

No vaccine or effective drugs are currently available for the prevention of bovine or canine neosporosis. Thus, control is primarily based on detection and culling of infected animals. Application of hygienic measures at farm level, and quarantine measures for imported animals can minimize the economic losses of neosporosis (Ortega-Mora et al., 2007; Sinnott et al., 2017).

Although cellular immune responses are required for controlling of *N. caninum*, both the innate and acquired immunities are effective against the infection. *N. caninum* can efficiently manipulate several subsets of immune cells including macrophages and T lymphocytes. Several previous studies revealed the critical role of IFN-γ in the direct killing of the parasite. These studies have been confirmed using different animal models, cell types and experimental approaches (reviewed by Aguado-Martinez et al., 2017; Nishikawa, 2017; Fereig et al., 2020). These *N. caninum*-host interactions are regulated by several key factors produced from the parasite.

Surface antigens of *N. caninum* tachyzoites regulate the process of adhesion and invasion of host cells. Two major surface antigens, NcSAG1 and NcSRS2, were identified and widely studied (Hemphill and Gottstein, 1996). NcSAG1 is anchored on the surface of tachyzoites by glycosylphosphatidylinositol (GPI) anchor (Howe et al., 1998), and NcSRS2 is also anchored on GPI (Nishikawa et al., 2002). Both antigens were observed to be down-regulated during tachyzoites to bradyzoites stage (Hemphill and Gottstein, 1996).

NcSAG1 is one of the most studied *N. caninum*, derived molecules in numerous studies of different purposes. It is extensively used as a potent antigen for *N. caninum* epidemiologic studies, diagnostic system, and vaccine studies (Sinnott et al., 2017; Fereig et al., 2020). These studies revealed the potential of NcSAG1 in stimulating cellular or humoral immune responses.

Accordingly, I will exploit the plenty of previous data, suggesting NcSAG1 as a pivotal molecule for neosporosis. To investigate the role of NcSAG1, a gene editing system based on CRISPR/Cas9 is used. Nishikawa et al. (2018) reported the successful establishment of knockout system for several genes using CRISPR/Cas9 in *N. caninum*. Herein, the deletion of NcSAG1 gene restricted the growth, proliferation and egress of the parasites *in vitro*. Moreover, my study demonstrated NcSAG1 as a determinant molecule for virulence of *N. caninum* either in non-pregnant or pregnant mouse models.

3-2. Materials and methods

Ethics statement

All experimental works were done in the current study, according to recommendations and procedures specified by the Guide for the Care and Use of Laboratory Animals of the Ministry of Education, Culture, Sports, Science and Technology, Japan. The methods were approved by the Committee on the Ethics of Animal Experiments at the Obihiro University of Agriculture and Veterinary Medicine (permission numbers 19-51, 19-56, 20-23, 20-27). Cardiac blood sampling was performed under general anesthesia of isoflurane, followed by euthanization by cervical dislocation.

Animals

Female and male BALB/c mice at 6 to 9-week-old, were purchased from Clea Japan (Tokyo, Japan). All used animals in this study, were maintained under specific-pathogen-free (SPF) conditions in the National Research Center for Protozoan Diseases at Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Japan. The used animals in current study were treated according to the Guiding Principles for the Care and Use of Research Animals published by the Obihiro University of Agriculture and Veterinary Medicine.

Parasite and cell culture

N. caninum (Nc-1 strain) was propagated Vero cells cultured in Eagle minimum essential medium (Sigma, St. Louis, MO) with additives 8% heat-inactivated FBS. For the purification of tachyzoites, parasites and host cell debris were harvested after washing with cold phosphate-buffered saline (PBS), and re-suspending the final pellet in cold PBS followed by passing through a 27-gauge needle and a 5.0-µm-pore size filter (Millipore, Bedford, MA).

Plasmid construction

CRISPR plasmids targeting the nucleotide (nt) 246 in the NcSAG1 gene were constructed for the insertion of the DHFR cassette. In brief, a single guide RNA (sgRNA) primer was designed using EuPaGDT website (http://grna.ctegd.uga.edu). A plasmid expressing CAS9 enzyme and sgRNA that targeting the uracil phosphoribosyl transferase (UPRT) gene of *T. gondii* (pSAG1::CAS9U6::sgUPRT) were obtained from Addgene (Cambridge, MA, USA). The Q5® Site-Directed Mutagenesis Kit (New England Biolabs, Ipswich, MA, USA) was used for the generation of CRISPR/CAS9 plasmid (pSAG1::CAS9-U6::sgNcSAG1) from the common plasmid (pSAG1::CAS9-U6::sgUPRT) through changing the UPRT-targeting sgRNA to NcSAG1 specific sgRNA using NcSAG1_246-gRNA primer (5'-AAA CAG GAC CGT CTG CCC GGC GG-3') and common primer for CRISPR/CAS9 plasmid that targeting *Neospora* genes (5'-AAC TTG ACA TCC CCA TTT AC-3').

Generation of NcSAG1 knockout parasites

An amplicon containing the homologous regions surrounding the pyrimethamine resistance dihydrofolate reductase (DHFR) cassette was prepared by PCR amplification using specific primes (DHFR-NcSAG1_246_1F; 5'-TAT CCA ACA AAC AGG ACC GTC TGC CAA GCT TCG CCA GGC TGT AAA-3', DHFR- NcSAG1_246_2R and 5'-TTT GAC CTC CGG ACT CCG CCG GGA ATT CAT CCT GCA AGT GCA TAG-3'). About 50 µg of the DNA of the previously constructed plasmid (pSAG1::CAS9-U6::sgNcSAG1) with 5 µg of DHFR cassette were used for transfection of wild type parasite (Nc-1) by electroporation for disruption of NcSAG1 gene. Then, the transfected parasites were grown in medium contains pyrimethamine antibiotic (1 µM) for two weeks for selection of stably resistant clones. Stably resistant clones were subsequently screened with PCR to ensure the correct integration of DHFR into the target gene locus. Further analyses using western blot (WB) and IFAT were applied to confirm the loss of the target gene in the PCR-positive clones.

N. caninum infection in mice

In order to assay the virulence of different parasite strains in mice, non-pregnant female (total n=18; 6 mice for each group) of BALB/c mice were intraperitoneally inoculated with Nc-1, NcSAG1KO clone #1 or NcSAG1KO clone #37 (1×10^6 tachyzoites/mouse). The mice were monitored for the survival rate, body weight and clinical score for 60 days post infection (dpi). The parasite loads in the brain tissue were measured from succumbed or sacrificed mice.

Regarding the pregnant mouse model, female BALB/c mice (9-weeks old) were housed with male mice at night time (one female with one male per cage) and checked in the early morning for the presence of seminal plugs in vagina. The first day on which a plug was noticed was designated as day 0 of gestation for each individual. All mice were challenged with 1×10^5 tachyzoites of Nc-1 or NcSAG1KO (clone #1) at 3 days of gestation (experiment 1, Nc-1; n = 5, NcSAG1KO; n = 6), and at

8 days of gestation (experiment 2, Nc-1; n = 6, NcSAG1KO; n = 6). Offspring survival rates were observed daily until 30 days after birth. The brain and uterus of all dams and the brain of some died and all surviving offspring at 30 days after birth (correspondent to 42-46 days after infection) were aseptically collected to determine the parasite burden.

Preparation of lysate antigen

Lysate antigen from purified tachyzoites of Nc-1 or NcSAG1KO was prepared as described previously (Liao et al., 2005). The obtained extract was filtered through a 0.45 μm low-protein binding Supor® membrane (Pall Life Sciences, Ann Arbor, MI, USA), and the concentration was measured using a bicinochoninic acid (BCA) protein assay kit (Thermo Fisher Scientific, Inc., Rockford, IL, USA).

Western blotting

Lysates from purified tachyzoites of *N. caninum* (Nc-1 or NcSAG1KO) were loaded by amount 1×10^6 tachyzoites/lane. Proteins samples were mixed with equal amount SDS gel reducing loading buffer 2× (62.5 mM Tris-HCl pH 6.8, 2% (w/v) SDS, 140 mM 2-mercaptoethanol, 10% (w/v) glycerol and 0.02% (w/v) bromophenol blue) and heated at 95ºC for 5 min. Denatured proteins were run on a 12% polyacrylamide gel for bands separation. The immunoblotting of samples was done by transferring protein bands in the gel to a nitrocellulose membrane (Whatman GmbH, Dassel, Germany). The membranes were washed once by PBS, and then were blocked with PBS containing 3% (w/v) skimmed milk (PBS-SM) overnight at 4ºC. In the following day, the membranes were incubated for 1 h at 37ºC with diluted anti-mouse monoclonal NcSAG1 (2C11) and NcSRS2 (1B8) antibodies (1:500 and 1:250, respectively) (Nishikawa et al., 2000) in 3% PBS-SM. Thereafter, the membranes were washed and incubated with horseradish peroxidase-conjugated anti-mouse IgG (1:4,000; Amersham Pharmacia Biotech, Piscataway, NJ, USA) diluted in PBS-SM, for 1 h at 37ºC. After further 3 washes, the protein bands were visualized using ECL™ western blotting detection reagents (GE Healthcare UK Ltd., Buckinghamshire, UK) by VersaDoc™ imaging system (Nippon Bio-Rad Laboratories, Tokyo, Japan) according to the manufacturer's recommendations.

IFAT

Vero cell suspension of 1 mL at 5×10^4 cells in MEM supplemented with 8% FBS was plated in each well containing a coverslip in 12-well plate, were incubated at 37°C in a 5% CO_2 atmosphere for 24 h. The cells were infected with purified Nc-1 tachyzoites at multiplicity of infection of 1:4 prepared in 1 mL of previously mentioned medium, incubated again at 37°C in an atmosphere of 5% CO_2 for 24 h, 48 h, or 72 h for assessment of infection, proliferation, or egress rate, respectively. Coverslips were washed 1 time with PBS then fixed with 3% paraformaldehyde in PBS (v/v) for 30 min at RT. After further washing with PBS once, 0.1% Triton X-100 in PBS was added to cells and

kept at RT for 10 min. After washing three times, the coverslips were incubated with 3% bovine serum albumin (BSA) in PBS (BSA-PBS) at RT for 1 h for blocking. As first antibodies, the coverslips were incubated with mouse monoclonal anti-NcSAG1 (Nishikawa et al., 2000) and rabbit polyclonal anti-NcGRA7 antibodies (Nishikawa et al., 2018) diluted 1:500 in 3% BSA-PBS at RT for 1 h. Then, coverslips were washed three times with PBS by 5 min interval, and were incubated with Alexa Fluor 488-conjugated goat anti-mouse IgG and Alexa Fluor 594-conjugated goat anti-rabbit IgG (Sigma) diluted 1:500 in 3% BSA-PBS for 1 h at RT. Nuclear DNA of all samples was marked with Hoechst 33342 (1:1,000 dilution, Thermo Fisher Scientific Inc., MA, USA). Finally, the coverslips were placed on a glass slide containing fresh drop from Mowiol (Calbiochem, San Diego, CA, USA), and the slides kept in dark for at least 2 h before examination using an All-in-one Fluorescence Microscope (BZ-9000, Keyence, Tokyo, Japan).

DNA isolation and real-time PCR analysis of N. caninum distribution

Genomic DNA was extracted from the brain and uterine tissues from tested mice as described formerly (Nishikawa et al., 2018), with slight modifications. Each tissue or organ was added in 10 volumes of extraction buffer (0.1 M Tris-HCl [pH 9.0], 1% SDS, 0.1 M NaCl, 1 mM EDTA) and 100 µg/ml proteinase K at 55°C. The DNA was purified with phenol-chloroform extraction and ethanol precipitation. The parasite DNA was then amplified with primers specific to the *N. caninum Nc5* gene. Amplification, data acquisition, and data analysis were performed in the ABI Prism 7900HT sequence detection system (Applied Biosystems), and the cycle threshold values (*CT*) were calculated. A standard curve was constructed using 10-fold serial dilutions of *N. caninum* DNA extracted from 10^5 parasites; thus, the curve ranged from 10 to 100,000 parasites. The parasite number was calculated from the standard curve.

Statistical analysis

All statistical analyses were performed with GraphPad Prism version 5 (GraphPad Software Inc., La Jolla, CA, USA). One-way analysis of variance (ANOVA) followed by the Tukey–Kramer test were used for group comparisons of parasite burden. Two-way ANOVA followed by the Tukey–Kramer test was used for estimating differences in body weight and clinical score results; data are presented as a standard error of the mean. The significance of the differences in survival was analyzed with a chi-square test or a log-rank test. The levels of statistical significance are presented with asterisks or letters and are defined in each figure legend. A P value < 0.05 was estimated as statistically significant.

3-3. Results

Characterization of NcSAG1*-deficient parasites by PCR, Western blotting and IFAT.*

Successful generation of the NcSAG1KO parasites was confirmed by PCR to detect the insertion of the DHFR cassette into the targeted site. Two clones from NcSAG1KO parasites were selected for further characterizations and analyses (NcSAG1KO#1 and NcSAG1KO#37). The amplification of the target gene was negative and the insertion of the DHFR cassette into the target gene was confirmed in deficient line (Fig. 1A). The absence of the target gene expression was also confirmed with western blotting (Fig. 1B). Anti-NcSAG1 monoclonal antibody detected 31.4 and 36.1 kDa proteins in the Nc-1 strain, but not in the *NcSAG1*-deficient clones #1 and #37, while anti- NcSRS2 monoclonal antibody could detect a 40.7-kDa protein in both Nc-1 and KO strains. Moreover, we confirmed the loss of target protein expression by IFAT. All parasite lines were confirmed by finding specific reaction against NcGRA7 while specific signal of NcSAG1 was only detected in Nc-1 parental strain (Fig. 1C).

In vitro *growth kinetic assays.*

The infection rates of *NcSAG1*-deficient parasite clones (#1 and #37 was 15% and 18%, respectively) in Vero cell at 24 h post-infection were lower than those of Nc-1 (25%) while this difference was not statistically significant (Fig. 2A). Moreover, I measured the numbers of parasites in parasitophorous vacuoles (PV) at 48 h post-infection between *NcSAG1*-deficient parasite and its parental strain (Fig. 2B). Significant reduction in number of tachyzoite was noticed in both *NcSAG1*-deficient clones compared to Nc-1 in the case of 4 and 16-tachyzoites/vacuole. This result suggests that *in vitro*-proliferation rates significantly affected by the loss of NcSAG1. The egress rate of parasites at 72 h post-infection was also measured (Fig. 2C). The deletion of *NcSAG1* gene significantly impaired the egress of the parasite. Overall, these results indicate the deletion of NcSAG1 delayed the growth kinetics of *N. caninum.*

Virulence assay in mice.

The virulence of the Nc-1 and NcSAG1KO#1 and NcSAG1KO#37 lines was also compared using non-pregnant female BLAB/c mice (Fig. 3 A-C). The *NcSAG1*-deficient lines showed lower virulence in relation to the parental strain Nc-1. This effect was obviously observed in the higher survival rate (NcSAG1KO#1: 66.7%, 4/6, NcSAG1KO#37: 66.7%, 4/6) in comparison to the parental strain Nc-1 (0%, 0/6). Interestingly, all Nc-1-infected mice were succumbed within 20 dpi while most of NcSAG1KO-infected mice survived along the course of experiment (60 days) (3A). Also, limited changes in the body weight and clinical score in the mice infected with *NcSAG1*-deficient lines than Nc-1-infected animals were recorded (Fig. 3B and 3C). Furthermore, the number of parasites in the brain tissues of died mice or those euthanized at 60 dpi was measured with quantitative real-time PCR (Fig. 3D). No statistically significant difference was found between all groups. Noteworthy, no any significant difference between both *NcSAG1*-deficient lines in any of the assayed parameters was seen.

The effects of NcSAG1 deletion on mouse offspring in pregnant mouse model.

Based on our previous results *in vitro* and *in vivo* non-pregnant mouse model, I assayed a role of NcSAG1 using only one *NcSAG1*-deficient clone (NcSAG1KO #1) in the pregnant mouse model. Two independent experiments were performed to elucidate the role of NcSAG1 in pregnant mouse model. In the experiment 1, female BALB/c mice were infected with Nc-1 (n = 5) and NcSAG1KO (n = 6) on the third day after vaginal plug confirmation. Uninfected mice were used as a control group. The conception rate was 33.3% (2/6) and 60% (3/5) of the group of mice infected with NcSAG1KO and Nc-1, respectively. The conception rate of the control group was estimated at 37% (3/8). In second trial, mice were infected with Nc-1 (n = 6) and NcSAG1KO (n = 6) at day 8 of pregnancy. The conception rate of infected groups was estimated at 50% for each group while the non-infected group showed an estimated pregnancy rate of 60%.

In both experiments, the survival rate and changes in the body weight of dams was evaluated (Fig. 4) until 30 days after birth. However, no significant differences were observed in any of these mentioned parameters. In addition, quantitative real-time PCR was applied for the detection of *Neospora* DNA in brain and uterine tissues of dams with no detected marked differences as well (Fig. 5). On the contrary, the effect of NcSAG1 was highly observed in the case of offspring from infected dams of the both experiments. In case of experiment 1, 33.3% (3/9) of the offspring from dams infected with NcSAG1KO survived for 30 days post-birth while all the pups (n=18) from dams infected with Nc-1 succumbed to infection within 20 days after birth (Fig. 6A). The parasite burden of the brain of survived or succumbed pups from dams infected with NcSAG1KO parasite was lower than those from Nc-1 infected dams, although there were no significant differences (Fig. 6C). In case of experiment 2, the survival rates of the pups from dams infected with the NcSAG1KO and Nc-1 until 30 days after birth were 33.3% (8/24) and 0% (0/24), respectively (Fig. 6B). *N. caninum* burden significantly decreased in the brain of the surviving offspring from dam infected with NcSAG1KO compared with offspring from Nc-1-infected dams (Fig. 6D). Together, the deletion of *NcSAG1* gene may decrease the neonatal mortality of the pups by reducing the parasite migratory ability of transplacental infection.

3-4. Discussion

Although numerous approaches to study gene function, knocking out of such gene is considered as the most efficient and reliable way. Recently, the CRISPR/Cas9 system has been successfully developed and evaluated against numerous *N. caninum* dense granule genes (NcGRA6, NcGRA7, NcGRA14 and NcCyp), and has led to several novel interesting data (Nishikawa et al., 2018). Expectedly, the role of NcGRA7 was superior to other above-mentioned genes as evidenced in its contribution in parasite virulence associated with higher inflammatory response and parasite burden in infected mice with Nc-1 compared with *NcGRA7*-deficient parasite (Nishikawa et al., 2018). However, NcSAG1 is one of most widely

investigated antigens of *N. caninum* in various research fields including diagnostics, vaccine developments and epidemiological studies. Such studies demonstrated the proficiency of NcSAG1 in the regulatory process of parasite adhesion and invasion of host cells, the interaction of the parasite with the immune system (Hemphill and Muller, 2015; Sinnott et al., 2016; Fereig and Nishikawa, 2020). Accordingly, the contribution of NcSAG1 in the pathogenesis of neosporosis can be undoubtedly extrapolated while the direct evidence for such information is lacking.

Despite the *N. caninum* infects canines as a definitive host and affects negatively the animal welfare, most of economic losses are attributable to induced abortion in cattle. In our previous study, we found that the anti-NcSAG1 antibodies are remarkably elevated in case of aborting cows against those infected but not-aborting ones (Abdelbaky et al., 2020). This report was also preceded by some other reports that recorded high antibody levels of NcSAG1 in aborted cattle (Huang et al., 2007; Hiasa et al., 2012; Takashima et al., 2013). Therefore, in the current study, the role of *NcSAG1* gene was investigated in the aspects of the virulence, abortion and vertical transmission of *N. caninum* through the generation of *NcSAG1*-deficient parasite.

The results of the growth kinetics of the parasite showed a significant decrease in the egress rate of the *NcSAG1*-deficient parasite comparing with the parental parasite. The *in vivo* results using non-pregnant mice revealed the efficient role of NcSAG1 in virulence of the parasite as indicated in the remarkable higher survival rate and lower clinical findings in *NcSAG1*-deficient parasite than Nc-1 parasites. These results are consistent with those obtained by Nishikawa et al. (2018) using NcGRA7KO parasite; the lacking of *NcGRA7* gene reduced the egress rate of the parasite *in vitro* and the parasite burden *in vivo* and thus preserved the life of the host.

An investigation in the possible role of *NcSAG1* gene in the abortion and/or vertical transmission of *N. caninum* was conducted in pregnant mouse model. Infection of pregnant mice was performed at two different time points (3 and 8 days after confirmation of vaginal plug) representing the early and the mid stages of pregnancy, respectively. However, the results were highly similar in both experiments either in dams or in their offspring. Survival rate of offspring from dams infected with *NcSAG1*-deficient parasite was 33.3% in both experiments. On the other hand, all offspring from Nc-1-infected dams were succumbed at 19 and 17 days after birth for experiment 1 and 2, respectively. This result was emphasized by the marked reductions of parasite burden in the brains of offspring from the infected dams with NcSAG1KO comparing to Nc-1. This result suggested that NcSAG1 was an essential molecule in the pathogenesis and vertical transmission of neosporosis in pregnant mice as well.

Numerous previous studies revealed the efficient potential of NcSAG1 in stimulating cellular or humoral immune responses **(**Reviewed thoroughly by Hemphill and Muller, 2015; Aguado-Martinez et al., 2017; Sinnott et al., 2017; Fereig and Nishikawa, 2020). However, the immunomodulatory effect

of NcSAG1 is more likely relevant to the cellular than humoral immune response (Kato et al., 2015; Marin et al., 2017). Some previous studies revealed the importance of Th-2-mediated immunity, especially IL-4 in maintaining the pregnancy in mice infected with *N. caninum,* on the contrary of IFN-γ (Gao et al., 1996; Kano et al., 2007). Based on such information and our data, perhaps NcSAG1 triggers hyper stimulation of cellular immune response, which subsequently induces detrimental effect on the infected host manifested in the inflammation and tissue damage. Nevertheless, further studies are required to explore the mechanism of reduction of virulence in non-pregnant mice and vertical transmission in pregnant mice after NcSAG1 disruption. Finally, my results strongly suggest the involvement of NcSAG1 in the pathogenesis of neosporosis either in non-pregnant or pregnant models of mice. Accordingly, our study presented NcSAG1 as a target molecule for vaccine development.

3-5. Summary

NcSAG1 is one of most widely investigated antigens of *N. caninum* in various research fields. Such studies demonstrated the proficiency of NcSAG1 in the regulatory process of parasite adhesion and invasion of host cells. Accordingly, the contribution of NcSAG1 in the pathogenesis of neosporosis can be undoubtedly extrapolated while the direct evidence is lacked. Herein, I provide the first successful attempt on gene disruption of NcSAG1, and novel data on invasion and virulence potentials of *N. caninum in vitro* and *in vivo*. The disruption of NcSAG1 gene was applied using CRISPR/Cas9 system, and confirmed by PCR, WB and IFAT test as NcSAG1KO. Then, I investigated the role of NcSAG1 in the growth kinetics of the parasite *in vitro*. The deletion of the NcSAG1 gene decreased the infection rate and significantly reduced the egress rate of the parasite. *In vivo* study using non-pregnant female BALB/c mice revealed significantly higher survival rate, lower body-weight change, and limited clinical score in the group infected with NcSAG1KO parasite compared to the parental strain (Nc-1)-infected group. Regarding vertical transmission model of mice, absence of NcSAG1 gene significantly enhanced the survival and greatly lowered the parasite burden in brain of pups. This study suggested NcSAG1 as a key molecule in the pathogenesis of *N. caninum*.

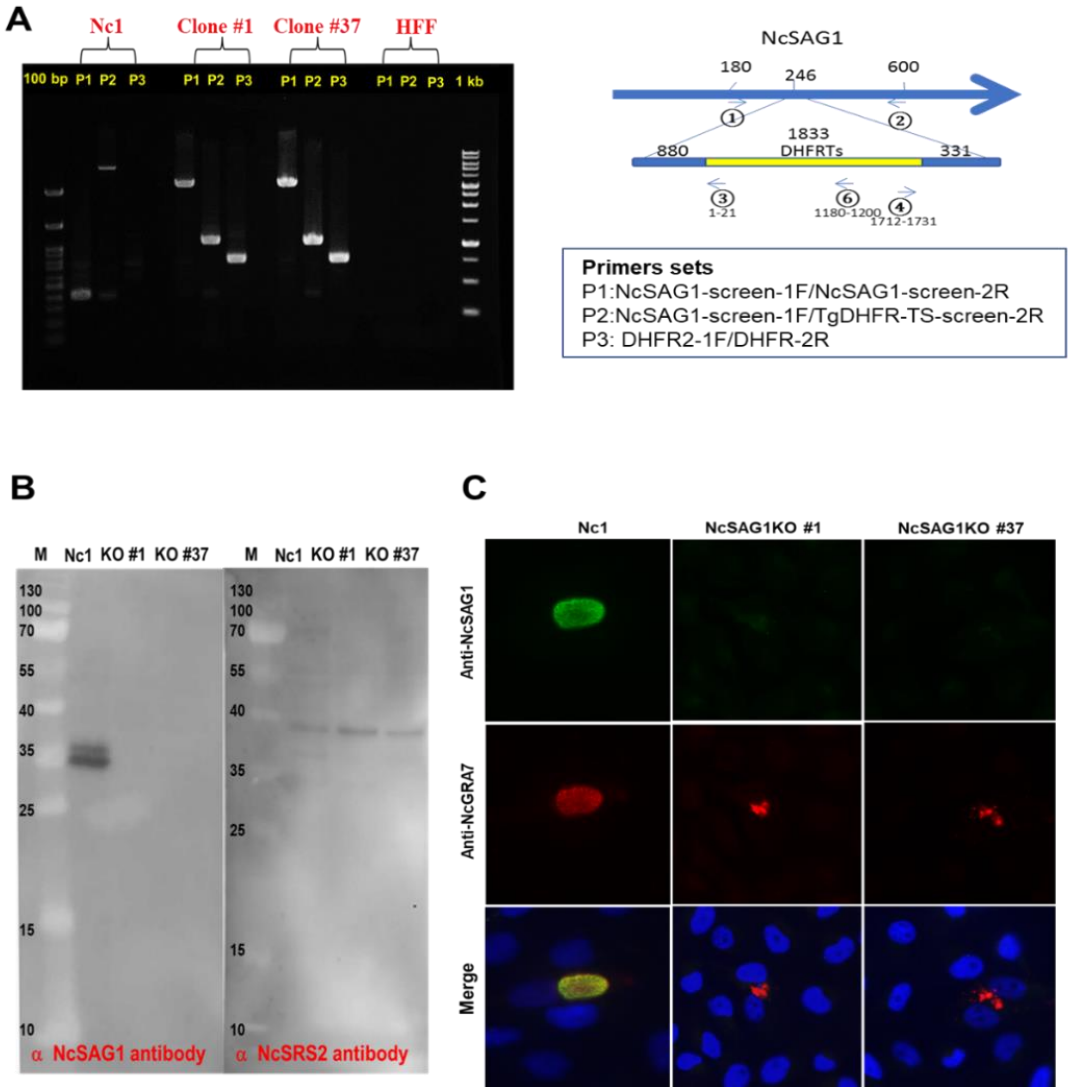


Fig. 12. Characterization of *NcSAG1*-deficient parasites (NcSAG1KO) using PCR, western blotting and IFAT. (A) PCR results demonstrated the gene disruption in NcSAG1KO clones (#1 and #37) compared with the parental line Nc-1. Product 1 (set P1) is the fragment amplified in the wild-type parasite (0.42 kb) while it is replaced by large fragment (1.83 kb) in the NcSAG1KO clones as a result of the insertion of DHFR cassette. Set P2; Product 2 (0.9 kb) and set P3; Product 3 (0.5 kb) provide evidence of homologous integration. (B) Western blotting of the parental strain Nc-1 and the NcSAG1KO#1 and NcSAG1KO#37. Anti-NcSAG1 monoclonal antibody detected two protein bands (31.4 and 36.1 kDa) in Nc-1 lysate but not in NcSAG1KO clones (left panel). Anti-NcSRS2 monoclonal antibody detected specific band (40.7-kDa) in all samples. M, molecular mass marker. Each figure panel represents photo taken from the same blot, including the marker. (C) IFAT analysis of Vero cells infected with Nc-1, NcSAG1KO#1 and NcSAG1KO#37 at 24 h post-infection. Cells were fixed and stained with mouse anti-NcSAG1 monoclonal antibody (green), rabbit anti-NcGRA7 (red), and Hoechst dye (blue).

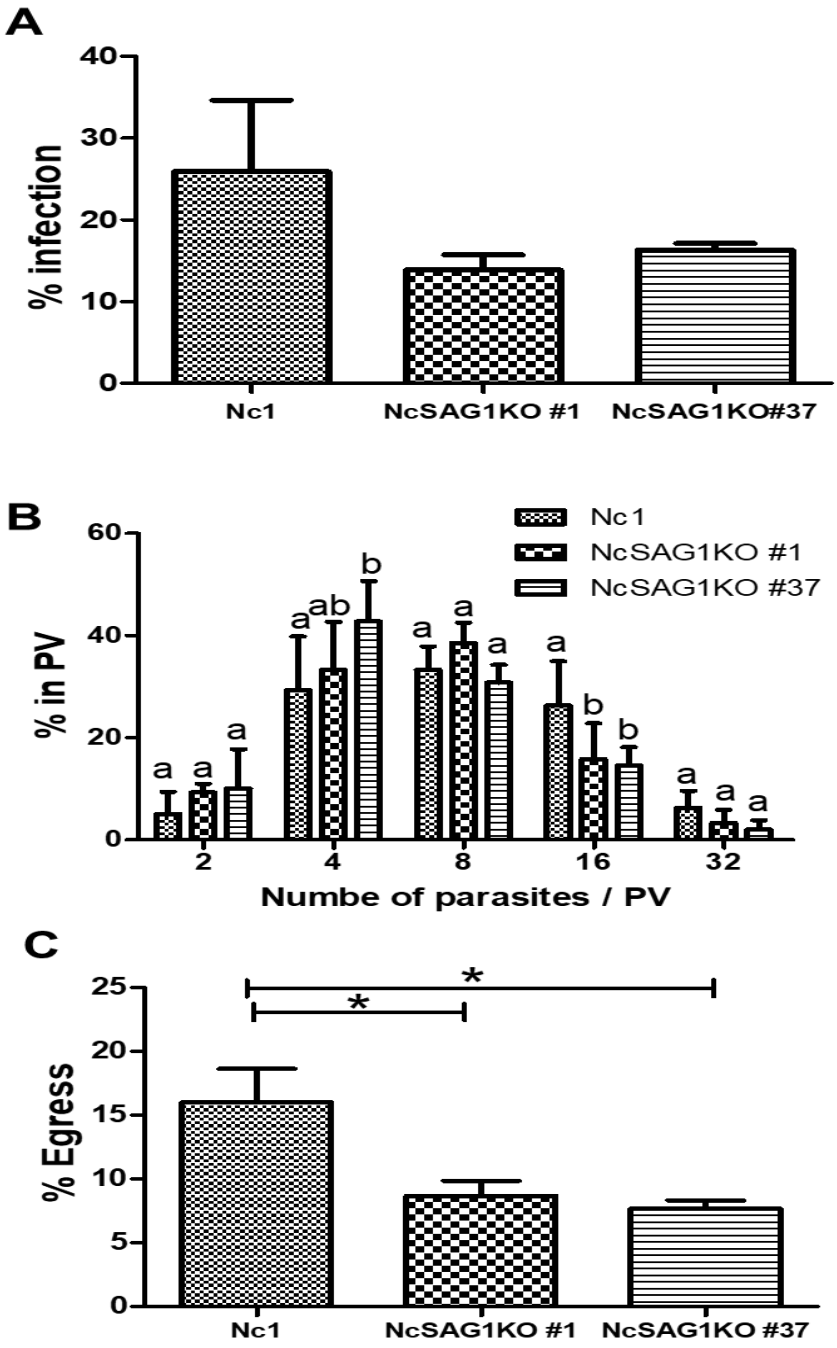


Fig. 13. Infection rate, growth, and egress assay. (A) Infection rates of the different parasite lines in Vero cells at 24 h post-infection. (B) Intracellular replication assay of the parasite lines in Vero cells at 48 h post-infection. (C) Egress rates of the different parasite lines in Vero cells at 72 h post-infection. Each bar represents the means ± the standard deviation (n = 4 for all groups), and the results represent two independent experiments with similar results. *, statistically significant differences relative to the value for Nc-1, according to *t*-test (A and C). Different letter represented significant differences in each parasitophorous vacuoles (PV) type according to two-way ANOVA and Bonferroni post hoc analysis ($P < 0.05$) (B).

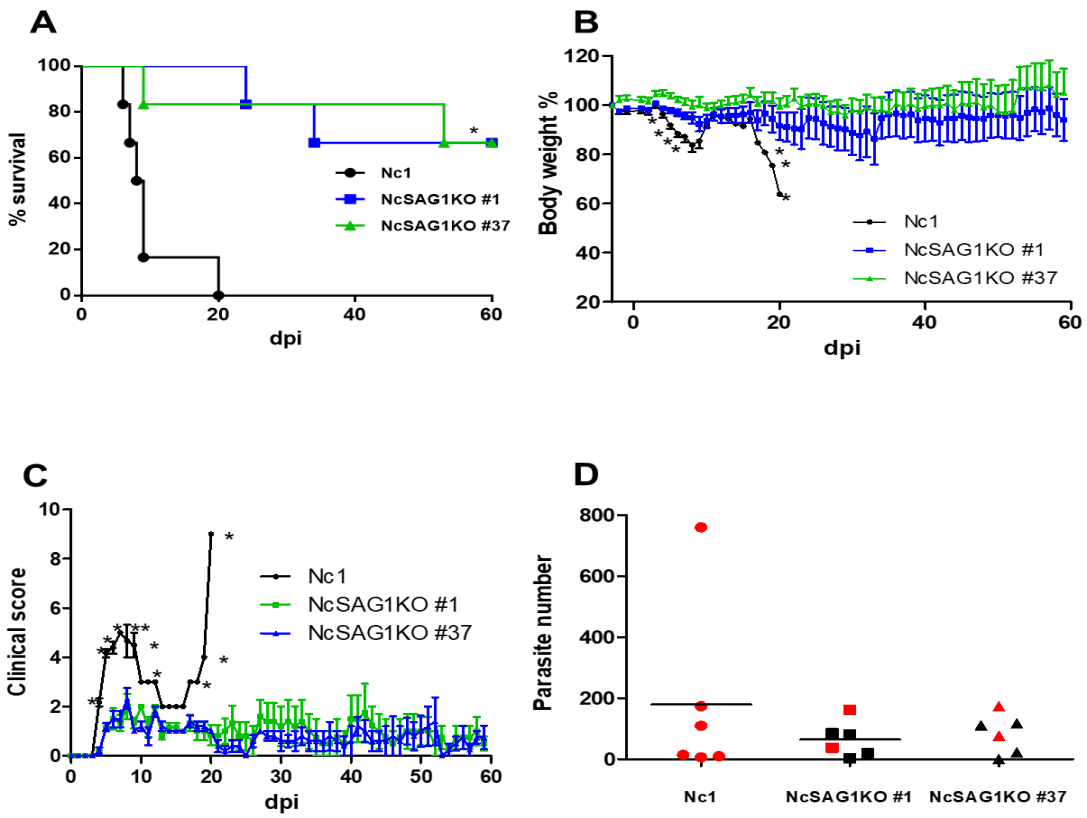


Fig. 14. Virulence assay in non-pregnant mouse model. BALB/c mice were infected intraperitoneally with a lethal dose (1×10^6) of *N. caninum* tachyzoites of the parental strain Nc-1, NcSAG1KO#1 and NcSAG1KO#37. The survival rates (surviving mice/total mice) were calculated for 60 days post infection (dpi). (A) Survival rates of BALB/c mice ($n = 6$ per group): Nc1, 0/6, 0%; NcSAG1KO#1, 4/6, 66.6%; NcSAG1KO#37, 4/6, 66.6%. The significance of the differences in survival at 60 dpi was analyzed with a chi-test (*, $P < 0.05$). Alteration of the clinical score (C) and body weight (B) were calculated as the means ± standard error (SEM) of clinical score and body weight values of all mice in a group from −2 to 60 dpi. The significance in change of body weight or clinical score was determined by two-way ANOVA plus Bonferroni post-test ($P < 0.05$). Asterisk (*) refers to a significant difference between the Nc-1 and NcSAG1KO infected groups. Only significant difference was observed between Nc-1 and against both KO clones NcSAG1KO#1 and NcSAG1KO#37. (D) Parasite numbers in the brains of the died and surviving mice until 60 dpi. The parasite burden was analyzed using one-way ANOVA plus Tukey–Kramer post hoc analysis, and no significant difference was observed between the different groups. Results for survival rate, clinical score, body weight and parasite burden are from one trial. The red-colored symbols in each column indicate that the samples have been collected from dead mice.

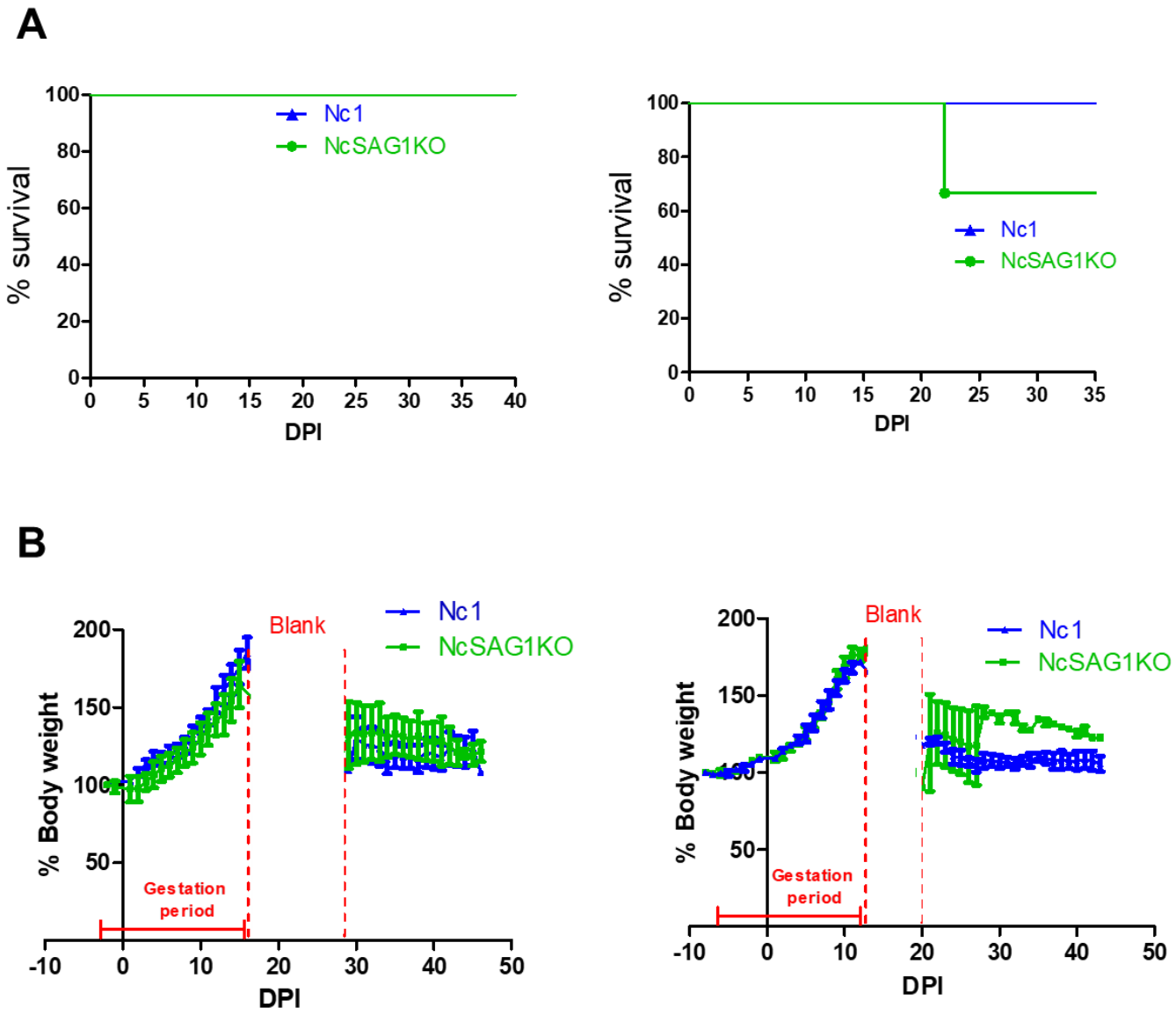


Fig. 15. Survival rate and body weight of dams. Alteration of the survival rate (A) and body weight (B) for experiment 1 (left) and experiment 2 (right) of infected dams with Nc-1 or *NcSAG1*-deficient parasite (NcSAG1KO#1) were calculated as the means ± standard error of survival rate and body weight values of all mice in a group from −2 to 47 dpi in experiment 1 or from −7 to 47 dpi in experiment 2. No significant differences were observed in the survival rate or in the body weight of infected dams using log-rank test or two-way ANOVA plus Bonferroni post-test, respectively.

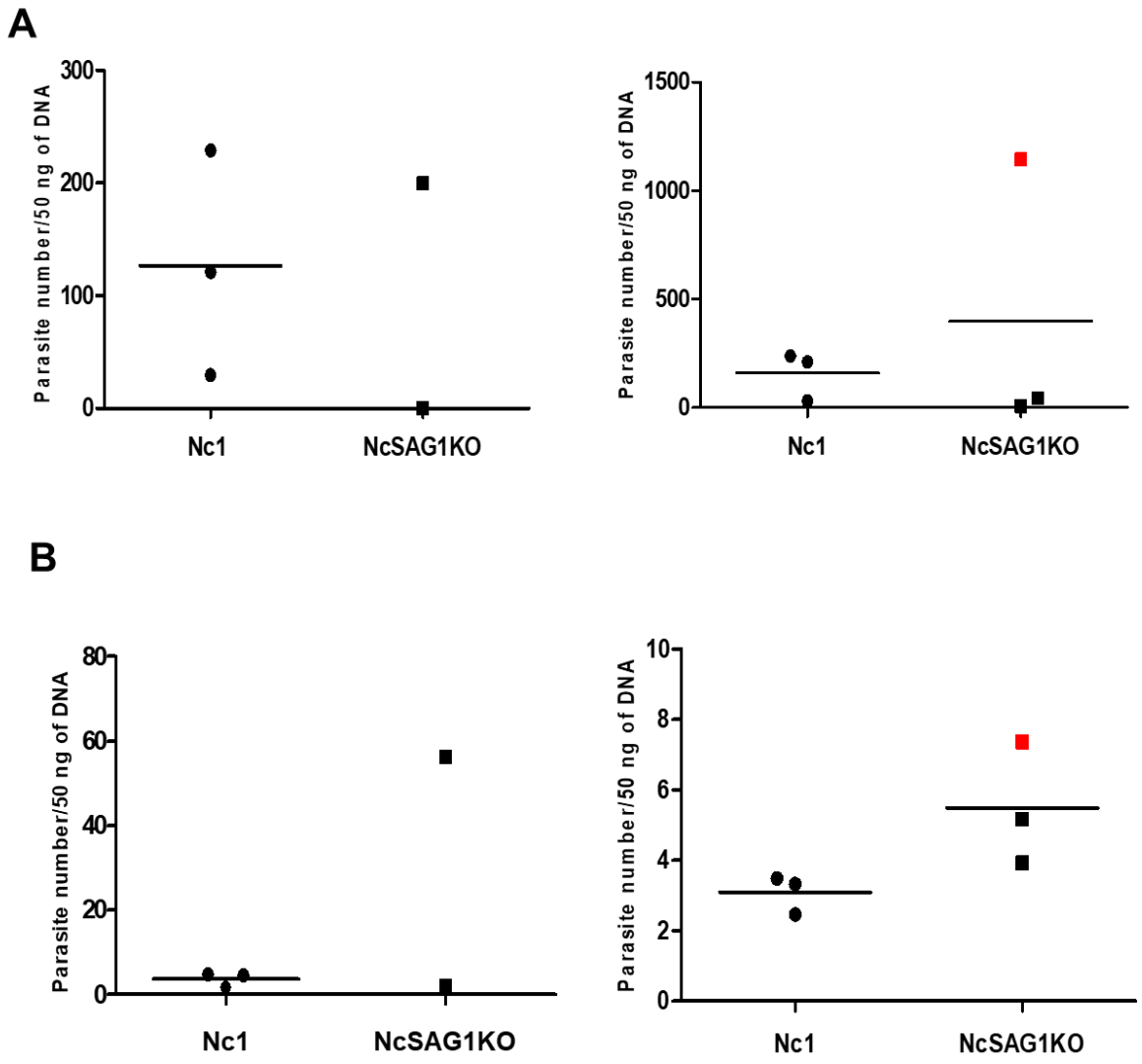


Fig. 16. Parasite burden in brain and uterus from pregnant dams. In pregnant mouse model, female BALB/c mice were infected intraperitoneally by non-lethal dose of Nc-1 or *NcSAG1*-deficient parasite (NcSAG1KO#1) (1×10^5). Female mice were infected either at 3 or 8 days after confirmation of vaginal plug and specified as experiment 1 or experiment 2, respectively. Parasite burden was measured the brains (A) and uterus (B) for experiment 1 (left) and experiment 2 (right) of the dead and surviving mice until 30 days after delivery. The parasite burden was analyzed using a Student's *t*-test analysis, and no significant difference was observed between the Nc-1 or NcSAG1KO infected mice. The red-colored symbols indicate the samples have been collected from dead mice.

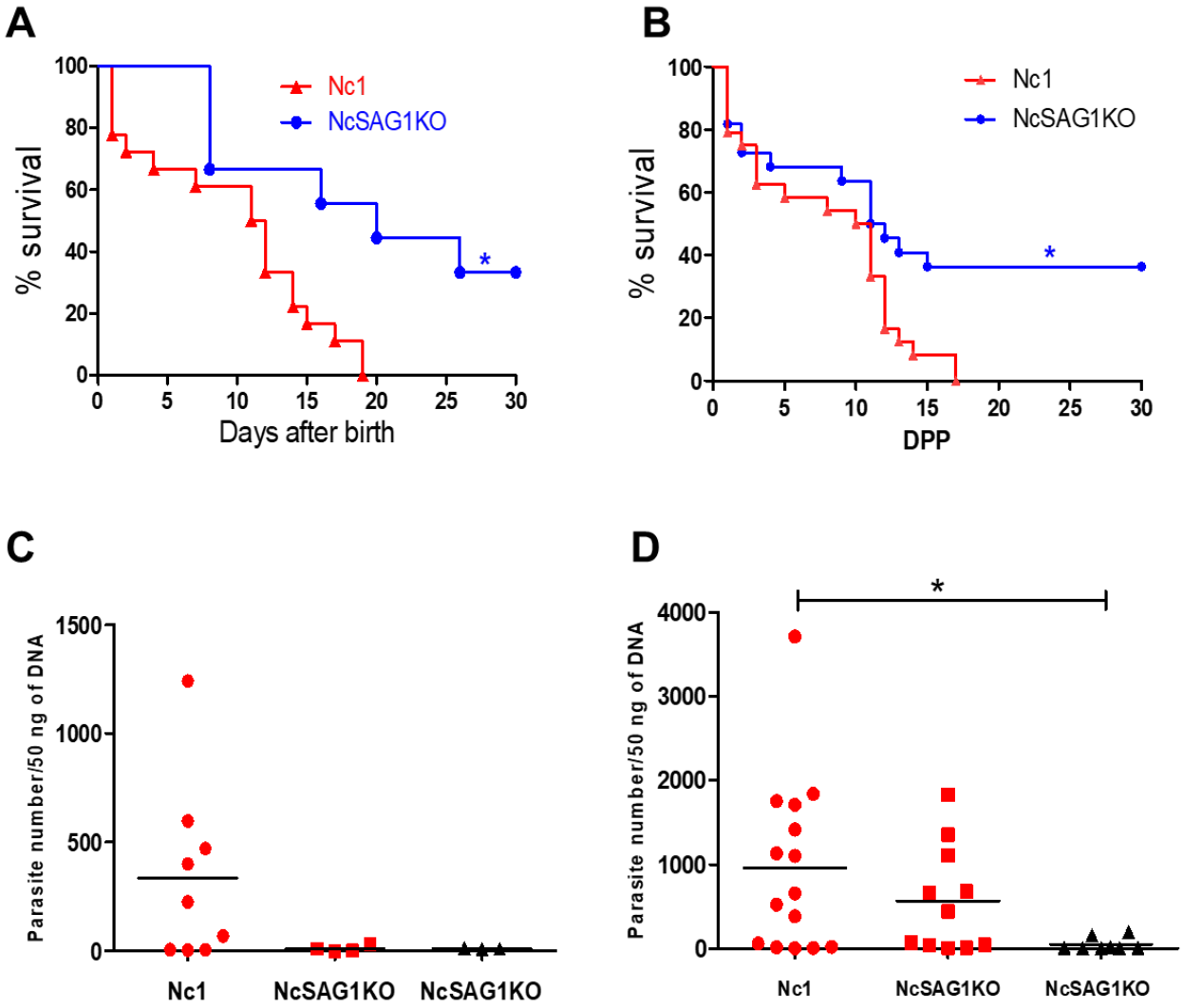


Fig. 17. Survival rate and parasite burden in offspring. Offspring from dams infected with non-lethal dose Nc-1 and *NcSAG1*-deficient parasite (NcSAG1KO#1) were monitored daily to calculate the survival rate and collect brains from dead ones. The survival rates of offspring were calculated until 30 days-after birth for experiment 1 (A) and experiment 2 (B). Survival curves were generated with the Kaplan–Meier method. According to the log-rank test, the difference was significant between Nc-1 and NcSAG1KO groups in both experiments (* $P < 0.05$). The parasite numbers in the brains of the died and surviving offspring at 30 days after birth were indicated for experiment 1 (C) and experiment 2 (D). Some brains of dead offspring could not be collected because of eating behavior by dam. *, statistically significant differences were observed with the One-way AVOVA followed by the Tukey–Kramer test. The red-colored symbols indicate that the samples have been collected from dead mice.

General discussion

Bovine neosporosis is a protozoan disease threatening cattle industry worldwide, which caused by the intracellular protozoan parasite *N. caninum*. The disease induces drastic economic losses as a consequence of abortion, neonatal mortality and congenital infections, imposing a high economic burden in cattle raising farms and associated industry. Although there are numerous trials with promising results, no commercially effective vaccines or pharmacological treatments are available for bovine neosporosis. Therefore, new targets for drug or vaccine development is urgently needed. Currently, accurate diagnosis for detection of infected animals followed by hygienic measures is the most available strategy for the control of *Neospora* infection. Removal of infected animal from the herd, culling of infected dams and their progeny from breeding, and quarantine of newly introduced animals into new herd should be applied (Dubey and Schares, 2006). Providing a new diagnostic tool by finding new diagnostic antigens can improve the current status of the diagnostic aspect of neosporosis. ELISA is the most common technique used at the herd level. ELISA is useful for a large-scale screening of samples, providing advantages with high specificity and sensitivity using specific antigens, such as surface antigens (SAGs) and dense granule proteins (GRAs). In particular, NcSAG1, NcSRS2 and NcGRA7 are more promising antigens for serodiagnostic purposes (Howe et al., 1998; Alvarez-Garcia et al., 2007).

NcGRA7 is an immunomodulatory antigen, which plays an essential role in the pathogenesis of neosporosis (Nishikawa et al., 2018). In addition, NcGRA7 contributes to the establishment of the PV in the host cell, and the active replication of the parasite (Hemphill et al., 1998; Aguado et al., 2010). Diagnostic performance of NcGRA7 was already reported against dog and cattle sera (Hiasa et al., 2012a; Takashima et al., 2013). A previous report showed that NcGRA7 recombinant protein is highly hydrophobic and a completely insoluble (Lally et al., 1996). This might be attributed to the containment of three hydrophobic regions in NcGRA7 protein, which decreased its solubility (Lally et al., 1997). Although removal of the signal peptide from the full-length of NcGRA7 gene markedly improved the expression in *Escherichia coli* (Hara et al., 2006), further investigation is required.

In the first chapter, further investigation was conducted to detect the relationship between solubility and antibody generation against different coding sequences of rNcGRA7. Additionally, I focused on the identification of the antigenic region of NcGRA7 protein in different animal species. As the first step, the entire coding sequence of NcGRA7 was split into different five fragments. Then, all the fragments were tested using negative control and sera from *N. caninum* experimentally infected mice, dog and cattle. The IgG1 and IgG2 antibody responses indicate the presence of Th2 and Th1-immune responses, respectively (Stevens et al., 1988). Three fragments, NcGRA7m, NcGRA7m3 and NcGRA7m4 showed antigenicity against IgG2 antibodies in sera from experimentally infected mice and dog, suggesting induction of cellular immune response. These results indicate the high diagnostic ability of the aforementioned fragments for the detection of *N. caninum* infection in mice and dog. In

naturally infected dogs, IgG2 levels were significantly higher in NcGRA7m3 than other fragments. On the contrary, humoral immune response was predominantly observed in experimentally and naturally infected cattle sera as indicated in higher level of IgG1 antibodies. Surprisingly, this effect was observed only in the NcGRA7m fragment. Accordingly, the fragmentation of NcGRA7 in cattle negatively affects the antigenic potential of NcGRA7, suggesting the involvement of the whole antigen structure in production of antibodies against NcGRA7 in cattle. My results for IgG1 with mature NcGRA7 is consistent with the findings of previous reports that also reported that NcGRA7 predominantly enhances humoral immune responses as indicated by higher IgG1 production (Hiasa et al., 2012b). Two fragments related to the C-terminal region, NcGRA7m5 and NcGRA7m6, were not antigenic in all tested animals.

This study identified NcGRA7m3 fragment as a novel antigenic region to detect *N. caninum*-specific antibodies in dog. This study also recorded differences in the antigenic properties of NcGRA7 among animal species. Different results between naturally and experimentally infected dogs may be due to different route of infection (Sanchez-Sanchez et al., 2018). While the variations in the types of immune responses among animal species may be attributed to the functional differences in immunoglobulin subclasses of the different hosts species (Cooper et al., 2006). Accordingly, the results of this chapter highlighted the variations of antibody production that must be considered in the development of diagnostic tools based on NcGRA7 antigen. Identification and characterization of specific antigen/antibody binding sites is the bases for development of effective diagnostics and vaccines. Thus, the mapping of antigenic regions of NcGRA7 will be useful in improving not only the diagnostic ability but also the vaccine potential. Eventually, the information provided in this study will assist in developing control strategies for *N. caninum.*

Diagnosis of *Neospora* abortion is essential for control management of the disease. Definitive diagnosis of neosporosis is based on the detection of *Neospora* antigen in tissues including the placenta of the aborted animals using IHC. Because of the high cost, invasiveness and low specificity and sensitivity of IHC test (De Merschman et al., 2005), a serological tool to support IHC is required. In chapter 2, I worked on developing a sensitive and specific test for estimation of *Neospora* abortion. Accordingly, this chapter aimed to establish a serological test for estimation of *Neospora* abortion through an antigenic comparison against five *N. caninum* potential diagnostic antigens, NcSAG1, NcGAR6, NcGRA7, NcGRA14, and NcCyP.

The diagnostic performance of the aforementioned antigens was evaluated using IgG-ELISA against sera from *N. caninum* experimentally infected mice and cattle. Three antigens, NcSAG1, NcGAR6 and NcGRA7, showed high performance against all tested sera, indicating their usefulness for the detection of *Neospora* infected animals. The other two antigens, NcGRA14 and NcCyP, were non-antigenic. Then, the performance of the highly diagnostic antigens, NcSAG1, NcGRA6 and

NcGRA7, was tested against sera from cattle had *Neospora* abortion confirmed by IHC and IFAT. High performance of NcSAG1 followed by NcGRA7 in the detection of bovine neosporosis was confirmed with significant higher antibody levels in IHC-positive samples comparing to IHC-negative samples. These results suggested NcSAG1- and NcGRA7-based ELISAs as useful diagnostic tools for estimation of *Neospora* abortion cases in cattle. These results were confirmed using cattle sera collected from aborted and non-aborted *Neospora* seropositive dams in a herd exhibited an epidemic abortion outbreak. Both of NcSAG1 and NcGRA7 antigens could detect *Neospora* aborted dams and distinguished them from *Neospora* infected animals statistically based on significant higher antibody levels in aborted cases. This result demonstrates the usefulness of NcSAG1- and NcGRA7-based ELISA as serological tools for supporting the final judgment of IHC in *Neospora* abortion.

The marked and sudden increase of specific antibodies against *N. caninum* during pregnancy is an indicator to *Neospora* reactivation, which considered as an alarm of *Neospora* abortion. Tracking the dynamics of NcSAG1- and NcGRA7-specific antibodies in sera of pregnant cattle showed an elevated level of their specific antibody at the last trimester of pregnancy. However, the highest number of animals with marked increases of antibody levels was observed in NcSAG1-specific antibody, particularly in the case of *N. caninum* seropositive animals at first sampling. On the other hand, only a few cases exhibited sero-conversion against NcGRA7. These results suggested the usefulness of NcSAG1 as a marker for *Neospora* reactivation during pregnancy. Periodic examination of maternal sera during pregnancy against NcSAG1 can predict abortion, and subsequently application of quarantine measures including the isolation of suspected animals, hygienic disposal of aborted fetus and maternal tissue will minimize the risk of disease transmission. The current result is similar to those obtained by Huang et al. (2007) that reported the utility of NcGRA7 and NcSAG1 as candidates for estimation of *N. caninum*-induced abortion in cattle.

Neurological disorders are the main signs of neosporosis in congenitally infected calves. My study showed the potential role of NcSAG1 and NcGRA7 based ELISA test in the detection of active infection of *N. caninum* in pregnant and aborted animals. Nevertheless, neurologically and non-neurologically symptomatic calves showed no significant differences in the levels of *N. caninum*-specific antibody. This finding indicates that levels of NcSAG1 and NcGRA7-specific antibodies are specifically associated with the condition of abortion rather than neurological symptoms. Accordingly, the results of this chapter using NcSAG1 indicated its superiority in evaluation of neosporosis, being a marker for parasite reactivation and estimation of *Neospora* abortion. It might suggest a potential role of NcSAG1 in the virulence and abortion of *N. caninum* infection.

Despite the significant economic damage resulting from the spread of the neosporosis in cattle and its drastic effect on animal welfare by causing paralysis in dog, the reports of neosporosis are limited. Previous studies are mainly focused on the seroprevalence using the well-defined diagnostic

proteins, and development of vaccine or pharmacological treatment. Identification of the virulence factors and the genes which mediate the infection especially in relevance to abortion or the vertical transmission of the parasite is essential to provide a new goal for treatment or vaccine development. The CRISPR/Cas9 is a successful genome editing technology for knockouts, overexpression and mutation of gene. This system is widely used for analysis of gene function in numerous apicomplexan parasites including *Plasmodium* (Ghorbal et al., 2014) *Toxoplasma* (Sidik et al., 2014) and recently *N. caninum* (Nishikawa et al., 2018).

Results of the chapter 2 suggested potential role of NcSAG1 as a virulence factor, which contributes to the abortion onset or the vertical transmission of *N. caninum*. In the chapter 3, I worked on the characterization of NcSAG1 gene to investigate its role on virulence, abortion and vertical transmission of *N. caninum* through the generation of NcSAG1KO parasite.

The disruption of NcSAG1 gene was performed using CRISPR/Cas9 technique and confirmed with PCR, western blot and IFAT in two different clones. The role of NcSAG1 gene in the invasion, multiplication and egress of the parasite in the host cell was investigated based on *in vitro* assay. The results of the growth kinetics of the parasite showed apparent but not significant decrease in the infection rate of NcSAG1KO than parental parasites. Significant results were obtained in the number of the vacuoles containing 16 parasites, and in the egress rate of the parasite in both NcSAG1KO clones comparing with the parental parasite. *In vivo* study using non-pregnant female BALB/c mice revealed higher survival rate of NcSAG1KO-infected mice (66.6%) while all mice infected with parental Nc-1 parasite were succumbed to infection at the acute stage of infection. Compared to the mice infected with Nc-1, significant lower body weight change and limited clinical score in the mice infected with NcSAG1KO was observed. Overall, this result suggested NcSAG1 as a key molecule in the pathogenesis of neosporosis in non-pregnant mouse model.

An investigation in the possible role of NcSAG1 gene in the abortion and/or vertical transmission of *N. caninum* parasite was conducted in pregnant mouse model. In the first trial, female BALB/c mice were infected with Nc-1 (n = 5) and NcSAG1KO (n = 6) on the third day after vaginal plug confirmation. Uninfected mice were used as a control group. The conception rate was 33.3% (2/6) and 60% (3/5) of the group of mice infected with NcSAG1KO and Nc-1, respectively. The conception rate of the control group was estimated at 37% (3/8). The survival rate of dams and their offspring was monitored for 30 days after birth. About 33% (3/9) of the offspring from dams infected with NcSAG1KO were survived for 30 days post-birth while, all the pups from dams infected with Nc-1 (n = 24) succumbed to infection within 20 days after birth. Quantitative real-time PCR was applied for the detection of *Neospora* DNA in brain tissue of dams and their offspring, in addition to uterine tissue of the dams. The parasite burden of the brain of survived or succumbed pups from dams infected with NcSAG1KO parasite was lower than those from Nc-1 infected dams while there were no significant

differences. In second trial, mice were infected with Nc-1 (n = 6) and NcSAG1KO (n = 6) at day 8 of pregnancy. The conception rate of infected groups was estimated at 50% for each group while the non-infected group showed an estimated pregnancy rate of 60%. As the survival rate for 30 days after birth, 33.3% and 0% of the pups survived from dams infected with the NcSAG1KO and Nc-1, respectively. *N. caninum* burden was decreased significantly in the brain of the surviving offspring from dam infected with NcSAG1KO compared with offspring from Nc-1-infected dams. Together, the deletion of NcSAG1 gene may decrease the neonatal mortality of the pups by reducing the parasite migratory ability of transplacental infection. These results strongly suggest the involvement of NcSAG1 in the pathogenesis of neosporosis either in non-pregnant or pregnant models of mice. Accordingly, my study presented NcSAG1 as a target molecule for vaccine development.

General summary

The development of serological tools for detection of *N. caninum* antibodies in different animal species is important to determine geographic distribution of the parasite. Such serological tools would assist in the application of appropriate control management, and subsequently minimize spread of the infection. In particular, serological detection of *N. caninum*-induced abortion in cows is a highly desirable approach to resolve the current problem of the diagnosis of neosporosis. Furthermore, the identification of *N. caninum* molecules responsible for the pathogenesis is also necessary to define new strategies for *Neospora* control. This can be accomplished by identification of efficient diagnostic antigens, developing potent vaccines, and discovery of drug targets. This study proposed the control of *N. caninum* infection via identification of potent antigens, development of serodiagnostic method for *N. caninum* related abortion, and understanding the pathogenesis of neosporosis.

In chapter 1, I mapped the antigenic regions of NcGRA7 diagnostic antigen against natural and experimental *N. caninum* infection in different animal species. The whole length of the antigen (217 amino acids) was split into five different fragments: NcGRA7m, NcGRA7m3, NcGRA7m4, NcGRA7m5 and NcGRA7m6. All the fragments were tested for their diagnostic performance using sera from three different animal species (mice, cattle and dogs) infected with *N. caninum* experimentally. Three fragments, NcGRA7m, NcGRA7m3 and NcGRA7m4, exhibited high antigenic performance against sera collected from experimentally infected mice and dogs. In sera from naturally infected dog, NcGRA7m3 fragment showed its superiority in comparison to other diagnostic fragments (NcGRA7m and NcGRA7m4). On the other hand, NcGRA7m fragment was the only antigenic region against sera from experimentally and naturally infected cattle. Two fragments, NcGRA7m5 and NcGRA7m6, which related to the C-terminal region, were unreactive. Accordingly, I reported the full length of NcGRA7 lacking the signal peptide, NcGRA7m, possessed the highly antigenicity in the detection of the antibodies against *N. caninum* in different animal species. This study highlighted the importance of species differences to develop NcGRA7-based diagnostic tools.

In chapter 2, a successful establishment of serological tool for accurate diagnosis of *Neospora* abortion was shown by comparison of the diagnostic performance of several *N. caninum* antigens in relation to abortion in field cattle. Firstly, five *N. caninum* antigens, NcSAG1, NcGRA6, NcGRA7, NcGRA14, and NcCyp, were expressed as recombinant proteins and evaluated by iELISA against sera of experimentally infected mice and cattle. Among them, three antigens, NcSAG1, NcGRA6 and NcGRA7 showed high antigenicity in experimentally infected animals. Next, the performance of NcSAG1, NcGRA6 and NcGRA7 was evaluated using sera collected from aborted cattle with confirmed neosporosis cases based on IHC assay. High correlations were observed between the results of the ELISAs and the IHC test where NcSAG1 and NcGRA7-based iELISA could detect 9/9 (100%) and 8/9 (88.9%) of IHC positive samples, respectively. In a herd with *Neospora* abortion outbreak, significant higher antibody levels against NcSAG1 and NcGRA7 were detected in aborted cows

comparing with non-aborting infected dams. Using serum samples collected from pregnant cows, high levels of anti-NcSAG1 antibodies were recorded at the last trimester of pregnancy indicates *Neospora* reactivation. However, no marked differences in the antibody levels against neither NcSAG1 nor NcGRA7 antigens were detected in neurologically symptomatic calves in comparison to non-symptomatic ones. This result suggests that the antibody levels against NcSAG1 and NcGRA7 were associated with *Neospora* abortion rather than neurological symptoms. This study represented NcSAG1 and NcGRA7-based iELISAs as serodiagnostic tools for detection and prediction of *N. caninum*-related abortion. Accordingly, this study can contribute significantly to reduce the hazard of *Neospora*-induced abortion in cattle and reduce the risk of disease transmission in the herd, if isolation of suspected animals and applying hygienic and quarantine measures prior to abortion were conducted.

In chapter 3, I focused on the functional characterization of NcSAG1 gene and its role in the virulence of *N. caninum*. My result in chapter 2 strongly suggests the contribution of NcSAG1 in the pathogenesis of neosporosis. Thus, I applied the generation of NcSAG1KO strain using CRISPR-Cas9 genome editing technology. PCR, IFAT and western blotting confirmed no expression of NcSAG1 in the knockout strain. The deletion of NcSAG1 gene significantly impaired the egress rate of the parasite *in vitro*. The result of *in vivo* experiment showed significant higher survival rate of mice infected with NcSAG1KO, accompanied with minimal changes in body weight and clinical scores, compared with its parental strain infection. Using a pregnant mouse model, the infection with NcSAG1KO during pregnancy resulted in increased neonatal survival and decreased parasite load in the brains of surviving newborn pups, then parental strain infection. Taken together, this study demonstrated NcSAG1 as a determinant gene in pathogenesis of *N. caninum*.

Finally, my study highlights usefulness of NcSAG1 and NcGRA7 as marker antigen for *N. caninum* related abortion in cows. In addition, NcSAG1 will be a potent molecule target for development of vaccine.

Japanese summary 和文要約

様々な動物種でネオスポラ抗体を検出できる血清学的手法の開発は、本原虫の地理的な分布を把握する上で重要となる。このような技術は適切な防疫対策を推進し、結果的に感染伝搬を最小限にすることができる。特に、ネオスポラ症の診断における現在の問題点を解消するために、ウシにおいてネオスポラ流産を判定できる血清学的手法が望まれている。さらに、病原性に関与するネオスポラ分子の同定は、ネオスポラの感染対策には必須である。有効な診断用抗原の同定、ワクチン開発、薬剤標的の発掘は、ネオスポラ感染の制御には重要である。本研究では、抗原の同定、ネオスポラ流産に対する血清診断法の開発、ネオスポラ症の病原性を理解することにより、ネオスポラ感染の対策方法を提案した。

第一章では、実験感染及び自然感染の各種動物に対し、血清診断用抗原 NcGRA7 の抗原性領域を決定した。全長 217 アミノ酸からなる抗原を５つの断片（NcGRA7m、NcGRA7m3、NcGRA7m4、NcGRA7m5、NcGRA7m6）として作製した。ネオスポラを実験感染させたマウス、ウシ、イヌの血清に対し、これら抗原断片の反応性を解析した。３つの抗原断片（NcGRA7m、NcGRA7m3、NcGRA7m4）は実験感染させたマウスとイヌの血清と高い反応性を示した。自然感染のイヌ血清では、NcGRA7m や NcGRA7m4 と比較して NcGRA7m3 の有効性が示された。一方、実験感染及び自然感染のウシ血清は NcGRA7m のみが反応性を示した。NcGRA7 の C 末端領域である２つの抗原断片（NcGRA7m5、NcGRA7m6）は抗原性が認められなかった。結果として、シグナル配列を除いた NcGRA7 の全長領域（NcGRA7m）がマウス、ウシ、イヌからネオスポラ抗体を検出することができた。本研究により、NcGRA7 を利用した診断法の開発には動物種を考慮する必要性が示された。

第二章では、複数のネオスポラ抗原を比較解析することにより、ネオスポラ流産の正確な診断のための血清学的手法の開発を行った。最初に５つのネオスポラ抗原（NcGRA6、NcGRA7、NcGRA14、NcCyp、NcSAG1）の組換えタンパク質を用い、実験感染させたマウスとウシの血清に対する反応性を ELISA で評価した。その中で３つの抗原（NcGRA6、NcGRA7、NcSAG1）が高い抗原性を示した。次に、免疫染色によりネオスポラ症の確定診断がされた流産牛の血清を用いて、３つの抗原（NcGRA6、NcGRA7、NcSAG1）の反応性を評価した。Nc SAG1 及び NcGRA7 を用いた ELISA はネオスポラ症のサンプルをそれぞれ 100%（9/9）と 88.9%（8/9）検出できた。ネオスポラ症が突発した１農家では、流産しなかったウシと比較して流産牛から高レベルの NcSAG1 抗体及び NcGRA7 抗体が検出された。さらに分娩前には NcSAG1 抗体レベルが上昇することが明らかとなり、分娩時におけるネオスポラの再活性化が示唆された。しかしながら、新生子牛においては神経症状の有無による NcSAG1 抗体及び NcGRA7 抗体のレベルに差は認められなかった。この結果により、NcSAG1 抗体及び NcGRA7 抗体のレベルが、神経症状ではなく流産と関連していることが示された。本血清診断法の導入により流産の発症前に感染疑いの個体を早期に摘発し防疫対策を講じることで、流産発生の危険を回避し牛群間の感染伝搬を減少させることができると考えられる。

第三章では、NcSAG1 の機能解析とネオスポラの病原性への関与に着目した。第二章の結果は、NcSAG1 が妊娠期におけるネオスポラ症の病態に関与することを

強く示唆している。そこで、CRISPR-Cas9 を利用したゲノム編集技術により NcSAG1 欠損原虫株（NcSAG1KO）を作製した。PCR、間接蛍光抗体法及びウェスタンブロット法により、NcSAG1KO における NcSAG1 の発現の消失が確認された。In vitro の実験で、NcSAG1 の欠損により原虫が感染細胞から脱出する能力が優位に減少していることが明らかとなった。In vivo の実験結果では、親株原虫の感染に比べて、NcSAG1KO 感染マウスで体重変動と臨床症状が軽度であり高い生存率を示した。妊娠マウスを用いた実験では、親株原虫の感染と比較して、NcSAG1KO 感染マウス由来の新生マウスでは生存率が上昇し、新生マウスの脳内原虫数も減少していた。従って、本結果により NcSAG1 がネオスポラの病原性因子であることが示された。

本研究により、ウシのネオスポラ流産のマーカー抗原として NcSAG1 と NcGRA7 の有用性が見出された。さらに、NcSAG1 はワクチン開発や創薬の分子標的になることが強く示唆された。

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