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Abstract of Thesis/Dissertation

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~~Master's~~/Doctoral Program in Animal and Food Hygiene

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

(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)

Abstract

Neosporosis is a disease of dog and cattle. The infection is caused by *Neospora caninum*, *Toxoplasma gondii*- like parasite. Currently, *N. caninum* is one of the major protozoan parasites that threaten cattle industry and affect animal welfare on the world level. Abortion and high neonatal mortality are the main signs of bovine neosporosis. Congenitally infected calves may exhibit some nervous signs including hyperextension in the hind limb. The domestic dog is the main final host, that carries all the developmental stages of the parasite. An infected dog shows different neurological signs such as paralysis which mainly affect the hind limb. Unfortunately, there is no safe medication or potent vaccines available to solve the problem of neosporosis.

Identification of the infected animal and the application of the hygienic measurement in the infected farms are the current applicable procedures for the control of neosporosis. 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 cases, and understand the pathogenesis of neosporosis via the identification of the virulence factors of the parasite

In chapter 1, I mapped the antigenic regions of the *N. caninum* dense granule 7 (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. The current study highlighted the importance of species differences to develop NcGRA7-based diagnostic tools.

In chapter 2 of my study, 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, four *N. caninum* dense granule antigens (NcGRA6, NcGRA7, NcGRA14, NcCyp) and *N. caninum* surface antigen 1 (NcSAG1), were expressed as recombinant proteins and evaluated by indirect enzyme linked immunosorbent assay (iELISA) against sera of experimentally infected mice and cattle. Among them, three antigens, NcSAG1, NcGRA7 and NcGRA6 showed high antigenicity in experimentally infected animals. Next, the performance of NcSAG1, NcGRA7 and NcGRA6 was evaluated using sera collected from aborted cattle with confirmed neosporosis cases based on immunohistochemical assay (IHC). 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 Clustered regularly interspaced short palindromic repeats-Cas9 (CRISPR-Cas9) genome editing technology. The polymerase chain reaction (PCR), indirect fluorescent antibody test (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 than parental strain infection. Taken together, this study demonstrated NcSAG1 as a determinant gene in pathogenesis of *N. caninum*.

In conclusion, my study highlights the species differences as an important factor in the development of a diagnostic tool based on NcGRA7 antigen. The usefulness of NcSAG1 and NcGRA7 as marker antigens for *N. caninum* related abortion in cattle was reported here. Additionally, this study identified NcSAG1 as an essential molecule in the virulence of *N.*

caninum parasite and in its vertical transmission. The NcSAG1 will be a potent molecular target for the development of a vaccine against neosporosis.