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INFORMATION

Hokkaido University conferred the degree of Doctor of Philosophy on September 25, 2014 to 8 recipients.
The titles of theses and other information are as follows:

Analysis of mast cells in the neonatal ovary of MRL/MpJ mice —unique immune cells participating in early follicular development—

Teppei Nakamura

Laboratory of Anatomy, Department of Biomedical Sciences, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060–0818, Japan

Mast cells (MCs) reside in most tissues and act as sentinel cells in both innate and adaptive immunity. In several mammalian species including human, MCs are also present in the adult ovaries, and previous studies indicated that MCs play some roles in reproductive functions. Although a few MCs are found in the neonatal ovaries of ICR and C57BL/6N mice, the functional relationship between MCs and the perinatal ovary is unclear. In this study, the author found numerous ovarian MCs (OMCs) in neonatal MRL/MpJ mice, analyzed them with follicular development, and clarified the factors affecting their appearance.

CHAPTER I: Among 11 different mouse strains, MRL/MpJ mice possessed the greatest number of OMCs. In MRL/MpJ mice, the OMCs were most abundant at postnatal day 0, and tended to localize adjacent to the surface epithelium. Immune cells such as macrophages, B cells, T cells, neutrophils and eosinophils were rarely observed in the ovary of MRL/MpJ and C57BL/6N mice at postnatal day 0. In MRL/MpJ mice, the number of OMCs within the oocyte nests was significantly higher than in the other strains, and some OMCs directly contacted the compressed and degenerated oocytes. In contrast, the OMCs localized outside of the oocyte nests rarely contacted with the primordial and primary follicles. In MRL/MpJ mice, the number of oocytes in the nest was significantly lower than in the other strains, and the number of oocytes showed a positive correlation with the number of OMCs. The gene expression of a MC marker also correlated with the expression of an oocyte nest marker. Furthermore, the expression of follicle developmental markers was significantly higher in MRL/MpJ mice than in C57BL/6N mice. These results indicate that the appearance of OMCs is a unique phenotype of neonatal MRL/MpJ mice, and OMCs would participate in the nest breakdown and contribute to the acceleration of the early follicular development in MRL/MpJ mice.

CHAPTER II: The author investigated the factors regulating the appearance of neonatal OMCs using the delayed parturition model and the progeny of the crosses between MRL/MpJ and C57BL/6N strains. In MRL/MpJ fetuses for which parturition was delayed until embryonic day 21.5, the number of OMCs was significantly higher than in age-matched controls at postnatal day 2. F1 neonates had less than half the number of OMCs than MRL/MpJ mice. Interestingly, MRLB6F1 had more neonatal OMCs than B6MRLF1, although they were distributed over comparable areas. Quantitative trait locus analysis using N2 backcross progeny revealed two significant loci on chromosome 8: *D8Mit343-D8Mit312* for the number of OMCs and *D8Mit86-*

D8Mit89 for their distribution, designated as *mast cell in the ovary of MRL/MpJ 1* (*mcom1*) and *mcom2*, respectively. Among MC migration-associated genes at *mcom1* locus, ovarian expression of chemokine (C-C motif) ligand 17 was significantly higher in MRL/MpJ than in C57BL/6N mice, and positively correlated with the expression of OMC marker genes. These results indicate that the appearance of neonatal OMCs in MRL/MpJ mice is controlled by environmental factors and filial genetic factors, and that the abundance and distribution of OMCs are regulated by independent filial genetic elements.

In mice just after birth, the oocyte nests break apart into individual cells and become primordial follicles. During this process, called nest breakdown, only a subset of oocytes survives and the remaining oocytes die. The author's results suggest that the numerous OMCs in MRL/MpJ mice participate in the nest breakdown, and contribute to progressed early follicular development, under the control of both the environmental factors and the genetic factors. Therefore, the author concluded that MC is a unique cell relating to the progression of early follicular development, and MRL/MpJ mice would be useful to clarify the close relationship between immune system and reproductive system through MCs.

The original papers of this thesis appeared in *PLOS ONE*, **8**: e77246 (2013) and **9**: e100617 (2014).

Development of the new protocol for habitat modeling of urban red fox to improve *Echinococcus multilocularis* control strategy

Takako Ikeda

Laboratory of Parasitology, Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060–0818, Japan

Echinococcus multilocularis Leuckart, 1863 is a parasite which will cause the human alveolar echinococcosis (HAE), one of the most serious helminthic zoonoses. HAE is spreading widely in the northern hemisphere and the number of cases has been increasing in recent years. The main transmitter of the parasite to human is the red fox, *Vulpes vulpes* Linnaeus, 1758. Deworming wild red foxes by baiting with the anthelmintic praziquantel is being established as a preventive technique to control the parasite. Improvement of the cost-benefit performance of baiting treatment is required urgently to maintain the efficacy of deworming.

In the present study, the efficient sites to be delivered the anthelmintic baits in urban area were determined by habitat modeling of red fox den sites. Habitat modeling of the generalist (the species which does not have any critical requirements for environmental resources) such as red foxes is considered to be difficult by existent methods, so the new protocol suitable for urban red foxes was developed.

The study was conducted in urban regions of Obihiro (about 59.8 km^2) and Sapporo cities (about 367.9 km^2) in Hokkaido, Japan, in which red fox populations have been established. The two cities have different degrees of urbanization. Sampling of fox dens location was conducted by thorough field exploring on the basis of the

information from citizens. A total of 35 fox dens in Obihiro study area (from 2002 to 2004) and 65 dens in Sapporo (from 2004 to 2007) were found. As against the points with dens "present", control points were dotted randomly on the analytical base map as "absence" data (120 points in Obihiro; 730 points in Sapporo). The base map was customized for analyzing red fox ecology inhabiting urban areas by modifying existent numerical information maps, residential maps, and aerial photographs, which consists of nine micro-habitat categories: "wide road", "narrow road", "occupied building", "vacant building", "water place", "riverbed", "farmland", "green covered area", and "blank space". These nine categories were set based on the previous reports on red fox habitat and to express the urban landscape. Den site selection modeling was conducted by use of the materials above for the red fox population in each of Obihiro and Sapporo study areas. The modeling protocol was designed to detect the best combination of the key environmental factors and the key spatial scale that foxes pay attention to (named "heeding range") when they select den sites, although the existent method can only detect the key environmental factors. All possible models were generated using logistic regression analysis, with "presence" or "absence" of fox den as the objective variable, and the percentages of the nine micro-habitat categories as predictor variables to detect the key environmental factors. This procedure was conducted for each of ten sizes of concentric circles (100–1000 m) from dens and control points to detect the most affecting circle size, that is, the key spatial scale. Out of all models generated, the best model was selected using Akaike's information criterion (AIC) inspection. This procedure was done in the each of the two study areas.

Established models suggest that requirements for denning are low percentages of wide roads, narrow roads, and occupied buildings, but high percentages of green covered areas within the circle of 500 m radius in Obihiro fox population; low percentages of wide roads, occupied buildings, but high percentages of riverbeds and green covered areas within 300 m radius in Sapporo. The difference in size of the key spatial scale between the two cities populations may come from the differences in their sensitivities to the surrounding environments. Both populations focused on the densities of wide roads, occupied buildings and green covered areas in common for their den sites. Accuracy of these models were inspected by area under the curve (AUC) of receiver operating characteristic (ROC) curve, and the values showed that those models have sufficiently high accuracy (AUC = 0.987 for Obihiro model; 0.995 for Sapporo model). Besides, prediction performances were evaluated by calculating the rates of concordance between observed and predicted values, and the rates were also sufficiently high (92.3% for Obihiro model; 99.2% for Sapporo model).

In conclusion, the den site selection models of urban red foxes have successfully been established, and the new modeling protocol has also been developed. The established model could determine the efficient sites for delivering baits, and it will improve the cost-benefit performance of the deworming by anthelmintic baiting campaign. Although it is generally considered that habitat selection modeling for generalist species is difficult, but the protocol developed in this study enabled it by use of the suitable variables for the target species. This modeling approach can be adopted for every type of habitat, including urban, suburban, rural, or primitive landscapes. The variation of the models shown in this study suggests the necessity of accumulating models for various types of cities in order to reveal the patterns of the model. It will enable us to perform rapid and efficient deworming campaign.

The original papers of this thesis appeared in *Parasit. Vectors*, **7**: 357 (doi: 10.1186/1756-3305-7-357) (2014).

Application of contrast-enhanced ultrasonography in diagnosis of canine pancreatic disease

Lim Sue Yee

Laboratory of Veterinary Internal Medicine, Department of Veterinary Clinical Sciences, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060–0818, Japan

Acute pancreatitis (AP) is a common disease of the canine exocrine pancreas and accurate noninvasive diagnosis is challenging. Contrast-enhanced ultrasonography (CEUS) is a major breakthrough for ultrasound imaging and can assess organ perfusion. This study was performed in three stages to determine the feasibility of using quantitative CEUS to diagnose canine AP.

Chapter 1 aimed to (1) characterize contrast-enhancement of the pancreas using bolus injection and continuous infusion of contrast agent and (2) to assess if continuous infusion can prolong pancreatic enhancement. CEUS of the pancreas were performed in eight dogs, and time-intensity curves were generated. Four perfusional parameters were measured for statistical analysis: time to initial up-slope (TTU), peak time (Tp), time to wash-out (TTW), and peak intensity (PI). Median pancreatic contrast-enhancement was prolonged by continuous infusion from 11 (range, 10 to 23) s to 205 (170 to 264; $P < 0.01$) s. Median PI was higher during bolus injection when compared to continuous infusion (100.9 [80.2 to 124.3] MPV versus 77.6 [58.2 to 99.5] MPV; $P < 0.05$). Prolonged continuous imaging was afforded by continuous infusion. PI was slightly lower in continuous infusion, but offered adequate imaging subjectively.

Chapter 2 aimed to investigate the feasibility of CEUS in detecting pancreatic perfusional changes in cerulein-induced canine AP. Six dogs received 2 hours of IV infusion with 7.5 μg/kg/h of cerulein diluted in saline. As control, all dogs received 2 hours of IV infusion of saline two weeks before cerulein infusion. CEUS of the pancreas were performed before (0 hour), and at 2, 4, 6 and, 12 hours after saline and cerulein infusion. Perfusion parameters TTU, Tp, TTW, PI, and area under the curve (AUC) were compared between saline and cerulein infusion. In cerulein-induced AP, PI increased at 2 and 4 hours when compared to 0 hour, and at 2, 4, and, 6 hours when compared to control. AUC increased at 4 hours when compared to 0 hour, and at 2 and 4 hours when compared to control. TTW was prolonged at 4 hours when compared to control. PI, AUC, and TTW can provide useful information in differentiating AP from normal pancreas. Cerulein-induced AP was characterized by prolonged hyperechoic enhancement on CEUS.

Chapter 3 aimed to investigate the feasibility of CEUS to detect pancreatic perfusion changes in naturally occurring pancreatitis. Twenty-three dogs diagnosed with pancreatitis were prospectively enrolled. Pancreatic CEUS were performed and perfusion parameters (TTU, Tp, TTW, PI, AUC) were compared to normal controls. Tp of the pancreatitis group was prolonged when compared to controls ($P < 0.001$). PI and AUC were increased when compared to controls ($P < 0.01$ and $P < 0.05$, respectively). CEUS can detect pancreatic perfusion changes in naturally occurring pancreatitis and was characterized by delayed peak with prolonged hyperechoic enhancement on CEUS.

In conclusion, pancreatic CEUS protocol was established in this study, and detection of pancreatic perfusion changes both in experimentally induced and naturally occurring pancreatitis were demonstrated. CEUS is potentially useful as a new noninvasive diagnostic tool in diagnosing naturally occurring canine pancreatitis.

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Studies on the antigenic characteristics of tick-borne flaviviruses and their application

Nozyechi Ngulube Chidumayo

Laboratory of Public Health, Department of Environmental Veterinary Sciences, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060–0818, Japan

TBEV and OHFV are both members of the TBE serocomplex although TBEV causes encephalitis while OHFV causes hemorrhagic fever syndrome. TBEV causes over 10,000 cases of encephalitis annually, with an endemic area extending from Western Europe to East Asia. The high public health significance of the virus necessitated the development of TBE vaccines. The development of a vaccine against OHF is economically unfeasible due to the lower prevalence of the disease. However, effective protection against OHF is required during outbreaks. The feasibility of a cross-protective flavivirus vaccine provides a cost effective method for OHF prevention.

The E protein of flavivirus is highly antigenic and is the major target for antibodies. The cross-neutralization of flaviviruses within a serocomplex is dependent on the degree of E protein similarity. Despite the obvious differences in disease manifestation, the two viruses share over 80% E protein homology. The high amino acid similarity suggested that a vaccine against TBE could cross-protect against OHF. In the chapter 1, the potential of commercially available TBE vaccines to protect against OHFV infection was investigated. Neutralization tests performed on sera from OHFV and TBEV-infected mice showed that neutralizing antibodies were cross-reactive. The GMT of antibodies against TBEV and OHFV from TBEV-infected mice were similar. However, the levels of anti-TBEV antibodies in OHFV-infected mice were significantly lower than the levels of anti-OHFV antibodies in the same animals. In mouse vaccination and challenge tests, the TBE vaccine provided 100% protection against OHFV infection. In addition, eighty-six percent of vaccinees seroconverted against OHFV following complete vaccination, and the GMT of neutralizing antibodies against OHFV were comparable to those against TBEV. These data suggest that the tick-borne encephalitis vaccine can prevent Omsk hemorrhagic fever virus infection.

TBEV is one of the most important vector-borne viral pathogens and the incidence of TBE has been increasing. Integrated surveillance of the vector and the virus in reservoir hosts and humans can identify TBE endemic risk areas and provide information for efficient prevention and control measures. Surveillance of the virus in reservoir species and humans requires cheap, reliable, and quick diagnostic tests. However, the production of commercial TBE ELISA kits is expensive as it requires the high biosafety facilities for the production of the inactivated whole virus antigen. The E protein is highly immunogenic and is therefore an important antigen for flavivirus diagnosis and vaccine development. In chapter 2, the applicability of E-Fc fusion proteins as antigens for TBE serodiagnosis was investigated. The E protein

ectodomain was fused to the Fc domain of rabbit IgG and the recombinant protein was expressed in mammalian cells. The recombinant E-Fc protein retained reactivity with both anti-TBEV and rabbit anti-IgG antibodies. The lack of cross-reactivity of the E-Fc antigen with mouse and human anti IgG antibodies suggested that the antigen could be useful in detecting anti-TBEV antibodies in multiple species. The E-Fc proteins were then used to develop an ELISA to detect TBEV antibodies. The E-Fc ELISA had high sensitivity and specificity in detecting TBEV antibodies in rodent and human sera. The results suggest this recombinant protein would be a good alternative to inactivated whole virus antigens in TBEV surveillance.

The original papers of this thesis appeared in *Microbiol. Immunol.*, **58**: 112 (2014) and *Diagn. Microbiol. Infect. Dis.*, **78**: 373 (2014).

Levels and effects of organochlorine pesticides and heavy metals in aquatic ecosystem from the Rift Valley region, Ethiopia

Yared Beyene Yohannes

Laboratory of Toxicology, Department of Environmental Veterinary Sciences, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060–0818, Japan

Organochlorine pesticides (OCPs) and heavy metals are ubiquitous and persistent contaminants with high bioaccumulation ability, and as a consequence high concentrations can be found in environmental and biota samples. In particular, species situated high on the food chain can accumulate very high concentrations of these environmental pollutants. OCPs and heavy metals are originated from natural (for heavy metals), and anthropogenic (for OCPs and heavy metals) sources. They have been associated with various toxic effects in humans and wildlife such as endocrine disruption, cancer, poisoning, serious illness and even death. Although the use of OCPs has been banned or restricted in developed nations, they are still being used for agricultural and public health purposes in developing countries like Ethiopia. Especially, one of the most controversial pesticide, DDT is being widely used in Ethiopia for agricultural and vector control. Furthermore, Ethiopia is burden with accumulated stockpiles of pesticides the so called “obsolete pesticides” since the first imported in the 1960s. The Ethiopian Rift Valley region which encompasses seven lakes is an important area for agricultural, commercial and industrial development of Ethiopia. At the same time it is one of the most environmentally vulnerable areas in Ethiopia. OCPs and heavy metals pollution in Ethiopia is anticipated mainly from anthropogenic sources.

In response to these concerns, the present study aimed to elucidate the bioaccumulation profiles and ecological risk assessment of OCPs and heavy metals from two Ethiopian Rift Valley Lakes—Lake Awassa and Lake Ziway. Twenty five surface sediment samples and three fish species; Tilapia (Oreochromis niloticus), Catfish (Clarias gariepinus) and Barbus (Barbus intermedius) from Lake Awassa, and five fish species; Tilapia, Zillii (Tilapia zillii), Carp (Carassius spp.), Catfishand Barbusand four bird

species; Hamerkop (Scopus umbretta), African sacred ibis (Threskiornis aethiopicus), Marabou stork (Leptoptilos crumeniferus) and Pelican (Pelecanus onocrotalus) from/around Lake Ziway were collected for this research study.

DDTs, HCHs, heptachlors, and chlordane compounds were the most predominant and ubiquitous residues. In general, the contamination levels of OCPs on both lakes were dominated by DDTs, attributing to their current use in vector control, illegal usage for agriculture, contamination from past usage and spills from obsolete pesticides. In sediment samples, the levels of DDT metabolites (DDE and DDD) were exceeded the sediment quality guideline values, and thus identified as chemicals of potential ecological concern in Lake Awassa. There were significant differences in OCPs levels among the studied fish species in the present study. The carcinogenic hazard ratio exceeded the threshold value of one for most of the studied fish species. Cumulative daily consumption of fish showed a potential concern on human health problem a lifetime cancer risk of greater than one in a million. High burden of DDTs was observed in all bird species under study. The level of DDE could pose deleterious effects on survival and/or reproduction in all bird species. According to the levels of heavy metals, they were found at low concentrations except to mercury (Hg), which exceeded the permissible limit (0.3 Hg μg/g ww) in B. intermedius fish species from Lake Awassa.

In general, my findings call for urgent action to reduce the level of OCPs' exposure and their effects on wildlife and human health. The low level of awareness in the study area and the public health and environmental consequence resulting from the misuse of pesticides is alarming. Therefore, there should be an integrated effort from governmental and non-governmental organizations in order to plan, tackle and control the use of pesticides effectively under the requirements of the Stockholm Convention, especially in relation to the misuse of DDT in agriculture. Although DDT is a low cost antimalarial tool, the possible adverse ecotoxicological effects through IRS must be carefully weighed against the benefits to malaria control. Routine ecotoxicological risk assessment of persistent organic pollutants in the Ethiopian Rift Valley region seems necessary.

The original papers of this thesis appeared in *Chemosphere*, **91**: 857–863 (2013), *Environ. Sci. Pollut. Res. Int.*, **20**: 8663–8671 (2013), *Ecotoxicol. Environ. Saf.*, **106**: 95-101 (2014) and *Environ. Pollut.*, **192**: 121–128 (2014).

The role of sika deer (*Cervus nippon yesoensis*) in the transmission of *Borrelia* spp. in Hokkaido, Japan

Kyunglee Lee

Laboratory of Wildlife Biology and Medicine, Department of Environmental Veterinary Sciences, Graduate School of Veterinary Medicine, Hokkaido University. Sapporo 060–0818, Japan

The intent of this research was to describe the role of deer in the transmission of different species of *Borrelia* spp. Sika deer (*Cervus nippon yesoensis*) are the hosts of ticks of various species and stages in Hokkaido. Deer overpopulation has caused problems with forestry and agricultural production. The potential of deer involvement in zoonoses is also being watched. In this study, deer caught in the nuisance control scheme and ticks collected from deer and the field were tested

to detect *Borrelia* spp.. These results were analyzed for the following two subjects.

Firstly, a survey was held related to a relapsing fever *Borrelia* sp. recently found in Hokkaido. A relapsing fever *Borrelia* sp. similar to *Borrelia lonestari* was detected from wild sika deer and *Haemaphysalis* ticks in the eastern part of Hokkaido, Japan. The total prevalence of this *Borrelia* sp. in tested deer blood samples was 10.6% using conventional PCR and real-time PCR methods. The prevalence was significantly higher in deer fawns compared to adults (21.9% and 9.4%, respectively). Additionally, there was a significant regional difference between two sampling areas, Shiretoko and Shibetsu with 17% and 2.8% prevalence, respectively. Regional differences were also found in tick species collected from the field and on deer. In the Shiretoko region, *Haemaphysalis* spp. were more abundant than *Ixodes* spp., while in Shibetsu, it was the opposite. Using real-time PCR analysis, *B. lonestari*-like was detected from 2 out of 290 adult *Haemaphysalis* spp. ticks and 4 out of 76 pools of nymphs. This is the first report of a *B. lonestari*-like organism in *Haemaphysalis* spp. ticks, and the first phylogenetic analysis of this *B. lonestari*-like organism in Asia. Based on this result, *Haemaphysalis* spp. are the most likely candidates to act as a vector for *B. lonestari*-like; furthermore, regional variation of *B. lonestari*-like prevalence in sika deer may be dependent on the distribution of these ticks.

The second survey was on Lyme disease borreliae in deer with an aspect different from the common vector-reservoir relationship. To determine whether and which types of borrelial spirochetes are extracted from *Ixodes persulcatus* ticks during feeding on sika deer, the infection rates of *Borrelia* spp. among *I. persulcatus* were compared between the two groups; the feeding ticks on deer and the questing flat ticks in the field. Lyme disease *Borrelia* spp. was detected in about 42% of adult and 6% of nymph questing ticks, while it was 3% and 1% from each group of feeding ticks, respectively. In contrast, the infection rate of *B. miyamotoi*, which belong to RF borreliae (sharing same vector), was not significantly different between groups, just below 1% among both feeding and questing ticks. Therefore, it could be said that sika deer may be the zooprophylactic host for ticks harboring Lyme disease borreliae in Hokkaido.

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Molecular epidemiological study of protozoan and other zoonotic diseases from two countries in Africa

Jesca Nakayima

Division of Collaboration and Education, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060–0818, Japan

Human African trypanosomiasis (HAT), also known as sleeping sickness, is a neglected tropical disease that impacts 70 million people distributed over 1.55 million km^2 in sub-Saharan Africa. *Trypanosoma brucei gambiense* accounts for almost 90% of the infections in central and western Africa, the remaining infections being from *T. b. rhodesiense* in eastern Africa. Furthermore, the animal diseases caused by trypanosomes inflict major economic losses to countries already

strained. The parasites are transmitted to the mammalian hosts through the bite of an infected tsetse fly. Additionally, zoonoses are infections or diseases that can be transmitted directly or indirectly between animals and humans. This study assessed the molecular epidemiology of human and animal trypanosomes, in addition to zoonotic pathogens in non-human primates in Zambia.

The first chapter of this thesis describes results of molecular epidemiological study on trypanosomiases which were carried out in two tsetse-infested areas of Ghana. The samples included tsetse flies, and cattle and pig blood, and were analyzed by using multiple polymerase chain reaction tests. *Trypanosoma vivax* was the most prevalent trypanosome species, followed by *T. congolense* and *T. brucei brucei*. Two subspecies causing HAT, *T. b. gambiense*, and *T. b. rhodesiense* were not detected in animals and flies in this study, which confirms that the country having been formally a HAT focus has been free of HAT since 2000. The results in this study may be reflected by the fact that *T. vivax* can be mechanically transmitted by biting flies in addition to biological transmission by tsetse fly, hence its distribution outside the tsetse fly belt of Africa.

The second chapter describes results on the genetic characterization of *T. vivax* strains from different geographical regions based on sequence comparison of Cathepsin L-like gene. *T. vivax* from Ghana clustered with West African and South American strains while *T. vivax* from Zambia clustered with East and Southern African strains. These results revealed genetic diversity of *T. vivax* in Africa.

In the third chapter, molecular epidemiological studies on zoonotic pathogens in non-human primates in Mfuwe in South Luangwa National Park, Zambia were carried out. This area is a HAT endemic focus with wildlife-livestock-human interface, hence the risk for zoonotic disease transmission is very high. Three species of zoonotic pathogens, *Rickettsia africae*, *Anaplasma phagocytophilum* and *Babesia microti* were detected among 9 pathogenic species/genera tested by PCR. These zoonoses detected in Zambia could be endemic in Zambian primates and possibly transmitted to humans but simply misdiagnosed as malaria due to their febrile nature.

Zoonoses in Africa are not just an African problem, since recent studies reveal an increase in these zoonotic infections in non endemic countries, especially in returning tourists from African national parks. Therefore, zoonotic disease control requires a multi-sectoral approach involving participants from the health, veterinary, entomology and environment professions because zoonosis transmission involves interaction between the pathogen, host, vector and environment.

The original papers of this thesis appeared in *Parasit. Vectors*, **5**: 217 (2012) and *Parasite*, **20**: 24 (2013).

Epidemiology, biological effects and treatment of free-ranging raccoon dogs (*Nyctereutes procyonoides*) infected with *Sarcoptes scabiei* in Kanagawa Prefecture, Japan

Nobuhide Kido

Nogeyama Zoological Gardens, Yokohama 220–0032, Japan

Sarcoptes scabiei (*S. scabiei*) is an ectoparasite that infests humans as well as domestic and wild mammals. *S. scabiei* infections are considered a major cause of mortality among wildlife, because wild animals cannot be treated. In addition, an *S. scabiei* outbreak might dramatically decrease the wildlife population. In Japan, *S. scabiei* infection has spread among raccoon dogs (*Nyctereutes procyonoides*), which are a native species. In addition, this situation is a concern for humans as well as domestic and wild animals that inhabit the same geographic area. Accordingly, a measure to deal with the spread of *S. scabiei* infection by raccoon dogs must be developed to ensure a healthy ecosystem. Thus, it is important to understand the characteristics and the effect of *S. scabiei* infections in the wild. The present study revealed the following three points associated with *S. scabiei* infection in raccoon dogs.

In chapter 1, the epidemiology of raccoon dogs infected with *S. scabiei* and the influence of *S. scabiei* infections on the population of other Carnivora were revealed. Three zoological gardens in Yokohama city have engaged in rescuing sick or injured wildlife since 1972, and the records of raccoon dogs rescued between 1981 and 2010 were examined. The number of raccoon dogs rescued due to car accidents or other reasons increased from 1989 onwards. The records likewise revealed that an *S. scabiei* outbreak occurred in 1993, and that the infection spread from the southern to the northern regions of Yokohama. Further, the total number of raccoon dogs rescued peaked in 1995, but declined thereafter. The high population density of the raccoon dog might have contributed to the radical spread of *S. scabiei* infection. Three Carnivora species, including raccoons (*Procyon lotor*), masked palm civets (*Paguma larvata*), and raccoon dogs inhabit Yokohama, and the habitats of these three species in urban areas are limited and partially overlap. In the Kanagawa prefecture, the raccoon is exterminated due to the alien species policy. Therefore, the raccoon population cannot be estimated in the present study. However, the masked palm civet and the raccoon dog are the targets of the wildlife rescue program, and their respective populations can be estimated. In the present study, the number of masked palm civets rescued gradually increased as the number of raccoon dogs rescued declined. Therefore, the raccoon dog and masked palm civet populations may be associated with each other in the wild. Consequently, the spread of *S. scabiei* in the raccoon dog might affect the population of the masked palm civet.

In chapter 2, it was revealed that debilitated raccoon dogs infected with *S. scabiei* exhibited abnormal hematological values compared to those of non-debilitated raccoon dogs infected with *S. scabiei*. Sarcoptic mange was considered a major cause of mortality. However, little is known about the hematological or serum biochemical values in clinically debilitated animals infected with *S. scabiei*. Accordingly, a comparison of hematological and serum biochemical values between severely debilitated and non-debilitated raccoon dogs infected with *S. scabiei* is presented in Chapter 2. The results indicated that the

white blood cell counts of both debilitated and non-debilitated raccoon dogs were increased. In addition, the total protein, albumin, glucose and calcium values of debilitated raccoon dogs were significantly lower than those of non-debilitated raccoon dogs. Conversely, the debilitated raccoon dogs had significantly higher aspartate aminotransferase, total bilirubin, blood urea nitrogen, and sodium, chloride and phosphorus values than the non-debilitated raccoon dogs. In particular, the increase in the blood urea nitrogen value was markedly dramatic. The results of the present study suggested that chronic infestations of *S. scabiei* debilitated raccoon dogs, and that the resultant hematological and serum biochemical abnormalities were caused by the infection.

In Chapter 3, effective treatments for *S. scabiei* infections in raccoon dogs were evaluated. Although the administration of ivermectin is the recommended treatment, many raccoon dogs in the Kanazawa Zoological Gardens have died due to *S. scabiei* infection, even after receiving a single ivermectin treatment. Since the hematological and serum biochemical abnormalities arose in raccoon dogs infected with *S. scabiei*, the effectiveness of treatment with an antibiotic for all raccoon dogs and fluids for the debilitated raccoon dogs in conjunction with ivermectin administration was evaluated. As a result, the number of animals that survived was significantly greater after the administration of ivermectin along with an antibiotic for all raccoon dogs, as well as following the administration of fluid therapy to the debilitated raccoon dogs infected with *S. scabiei*. During the initial period, treatment to improve the general clinical condition was required prior to deworming treatment for *S. scabiei*.

In conclusion, the current study revealed the epidemiology of raccoon dogs infected with *S. scabiei* infection in Kanagawa Prefecture, Japan. Moreover, chronic infection of *S. scabiei* debilitated raccoon dogs, and caused hematological and serum biochemical abnormalities. Based on the results, an effective treatment to improve the survival rate of raccoon dogs infected with *S. scabiei* was established. The results provided valuable information to consider when evaluating *S. scabiei* infections in raccoon dogs, and contributed to the establishment of a methodology to ensure preservation of a healthy ecosystem.

The original papers of this thesis appeared in *Parasitol. Int.*, **60**: 425–428 (2011), *Vet. Parasitol.*, **191**: 102–107 (2013) and *J. Vet. Med. Sci.*, **76**: 1169–1172 (2014).

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INFORMATION

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Genetic and pathogenetic diversity of fowl glioma-inducing viruses

Sayuri Nakamura

Laboratory of Comparative Pathology, Department of Veterinary Clinical Sciences, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060–0818, Japan

Fowl glioma is histologically characterized by multiple nodular astrocytic growths with disseminated non-suppurative encephalitis. In 1995, the first case of fowl glioma in Japan was found in Japanese native fowls (*Gallus gallus domesticus*). Subsequent experimental studies demonstrated that the disease is caused by fowl glioma-inducing virus (FGV) prototype, belonging to subgroup A of avian leukosis virus (ALV-A) and that this strain could also induce cerebellar hypoplasia and perineurioma in chickens.

The *env* gene is known to play important roles in the oncogenicity and tissue tropism of ALV. However, whether FGV variants with mutations and deletions of nucleotides of the *env* have neurotropism and/or gliomagenicity remains unclear. Thus, the aim of the present studies was to clarify the pathogenicity of FGV variants. In addition, pathological and molecular biological analyses on cerebellar anomaly in chickens and cardiac abnormality in Japanese native fowls were performed, because these conditions are suspected to be due to ALV infection.

In Chapter I, the complete nucleotide sequences of four FGV variants, including Tym-43, U-1, Sp-40 and Sp-53, were determined and their pathogenicity was investigated to clarify whether or not these strains have the ability to induce brain lesions. The sequences of the surface (SU) proteins encoded by *env* of these viruses had 85 to 95% identity with the corresponding region of FGV prototype. Next, SPF chickens were inoculated with these variants as well as the chimeric virus RCAS (A)-(FGV*env*SU), constructed by substituting the SU region of FGV prototype into the retroviral vector RCAS (A). The four variants induced glioma and cerebellar hypoplasia. In contrast, RCAS (A)-(FGV*env*SU) provoked only mild non-suppurative inflammation. These results suggest that the ability to induce brain lesions similar to those of the FGV prototype is still preserved in these FGV variants and the *env*SU is not a crucial determinant to the glioma-inducibility of FGVs.

In Chapter II, the relationship between intracerebral replication of Sp-53, which has induced the most severe lesions in the preceding chapter, and astrocytic growth in the early infection phase was investigated. Replication abilities of two ALV strains, Sp-53 and RCAS (A), were compared in the brains of SPF chickens at 35 days of age. Sp-53 replicated faster than RCAS (A), and the histological score and the RNA level of IL-1β were increased depending on the level of intracerebral viral RNA. Up-regulation of IL-1β was also demonstrated in primary cultured astrocytes. These results imply that the astrocytic growth in this phase is enhanced through the autocrine/paracrine production of IL-1β in the

FGV-infected astrocytes.

In Chaper III, cerebellar anomalies from seven SPF White Leghorn chickens (*Gallus gallus domesticus*) were examined to clarify the morphological characteristics of affected cerebellums and the relationship between them and ALV infection. Grossly, the cerebellums showed disorganization of cerebellar folia and the macroscopic change was closely similar to that of FGV-induced cerebellar hypoplasia. Histologically, islands of heterotopic cortex were distributed from deeper cortices to the medulla in the cerebellum. The characteristic lesions were composed of randomly admixed components of cerebellar cortex, including soma of Purkinje cells, dendrites of Purkinje cells in the molecular layer and granule cells. Immunofluorescence analysis revealed Purkinje cells with haphazardly extended dendrites in the foci. Chicken parvovirus and ALVs were not detected in the affected birds by PCR. From these results, the lesions were diagnosed as cerebellar dysplasia and quite different from FGV-induced cerebellar lesions.

Unusual cardiomyocyte hypertrophy with mitosis was recently observed in Japanese native fowls infected with ALVs. The affected hearts were evaluated by histopathology and immunohistochemistry, viral isolation, viral genome sequencing and experimental infection in the last chapter. There were non-suppurative myocarditis and abnormal cardiomyocytes characterized by hypertrophic cytoplasm and atypical large nuclei in the affected hearts. Nuclear chains, mitoses and matrix inclusions were frequently noted in these cardiomyocytes. ALVs were isolated from all affected birds and phylogenic analysis of *env*SU showed that isolates were mainly classified into two different clusters. *In ovo* experimental infection with two of the isolates was demonstrated to cause myocarditis and cardiomyocyte hypertrophy similar to those in the naturally occurring lesions. These results indicate that ALVs cause cardiomyocyte abnormality in chickens.

The present studies indicate that there is genetic and pathogenetic diversity among FGVs and the related ALVs, which still spread among Japanese native fowls in Japan.

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INFORMATION

Hokkaido University conferred the degree of Doctor of Philosophy on March 25, 2015 to 8 recipients.
The titles of theses and other information are as follows:

Studies on molecular pathogenesis of murine autoimmune glomerulonephritis—an early sign to the progression of chronic kidney disease indicated by injured podocytes—

Junpei Kimura

Laboratory of Anatomy, Department of Biomedical Sciences, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060–0818, Japan

Recently, the growing number of elderly individuals has led to the worldwide increase in both humans and animals with chronic kidney disease (CKD). In this thesis, the author investigated the molecular pathogenesis of chronic glomerulonephritis (GN), which is one of the major CKD primary diseases, to clarify the pathological features, genetic basis, and the diagnostic or therapeutic targets of CKD.

In Chapter 1, the author investigated BXSB/MpJ-*Yaa* (BXSB-*Yaa*) mice, a representative model for autoimmune GN due to mutated *Yaa* locus on chromosome (Chr.) Y. BXSB-*Yaa* mice developed glomerular lesions (GLs) and tubulointerstitial lesions (TILs) characterized by the expansion of mesangial matrix, proliferation of mesangial cells, dilated tubules by urinary casts, and perivascular cell infiltration. In BXSB-*Yaa* mouse urine, the cell numbers as well as the mRNA of cell specific markers for podocytes, distal tubules (DTs), and collecting ducts (CDs) increased with disease progression. Further, injured DTs and CDs produced C3 mRNA and protein, and its mRNA was detected BXSB-*Yaa* mouse urine at high rates. Briefly, in CKD condition, injured epithelial cells of the glomerulus, DT, and CD were dropped into the urine.

Recent studies have indicated that CKD often begins with GLs including blood urine barrier (BUB) disruption, leading to subsequent TIL. The author next focused on the injuries of podocyte, a glomerular epithelial cell regulating BUB integrity. In addition to BXSB-*Yaa* mice, B6.MRL-(*D1Mit202-D1Mit403*) (B6.MRL) mice were investigated as autoimmune models. Both GN models developed membranous proliferative GN with albuminuria and elevated serum anti-double strand DNA antibody (anti-dsDNA) levels. Ultrastructural analysis revealed the abnormal ultrastructural morphology of podocytes. Further, the prominent decreases of mRNA and protein levels of podocyte functional factors were observed in the glomerulus of both models, and they negatively correlated with urinary albumin ratios (uACRs). These results strongly emphasized the clinicopathological importance of podocyte injuries with altered function and morphology in CKD.

In Chapter 3, the author explored the genetic factors affecting CKD pathogenesis, especially focusing on GL of BXSB-*Yaa* mice. Surprisingly, female BXSB/MpJ (BXSB) mice showed the increase of serum anti-dsDNA antibodies as well as membranous proliferative GN with aging in despite of *Yaa* absence. Male BXSB mice developed neither autoimmune symptom nor GN.

From the comprehensive gene expression analysis, the author found that the expression of autoimmune GN candidate genes on the telomeric region of Chr.1, such as *Fcgr3* and *Ifi202b*, drastically upregulated in the glomerulus of male BXSB-*Yaa* mice compared to male BXSB mice. Their renal expression was also significantly upregulated in female BXSB mice. These results suggest that that sex-differences in immunity might affect GN pathogenesis of BXSB strain, and that *Yaa* contributes the progression of CKD by enhance the expression of autoimmune GN candidate genes localizing to the telomeric region of Chr.1 on BXSB-type genome.

The Chapter 4 focused on Toll-like receptor (TLR) family coded on *Yaa* locus to identify the early diagnostic and therapeutic targets for CKD. The TLR family plays a crucial role in local autoimmune response. The exhaustive expression analysis for TLR family genes showed the *Tlr8* overexpression in glomerulus of BXSB-*Yaa* and B6.MRL mice. TLR8 protein and mRNA localized in podocytes of mouse as well as human kidney. The isolated-glomerular *Tlr8* expression were significantly correlated with those in podocyte functional markers negatively and uACR positively in BXSB-*Yaa* mice. Further, the glomerular and serum levels of miR-21, a putative miRNA ligand of TLR8, were significantly higher in BXSB-*Yaa* mice than in BXSB mice. The urinary levels of *Tlr8* mRNA were also significantly higher in BXSB-*Yaa* mice than in BXSB mice. These results strongly suggest that the overactivation of TLR8 contributes to progression of podocyte injury in GN. Altered levels of urinary *Tlr8* mRNA may thus reflect podocyte injury, and TLR8 would be novel target of diagnosis and therapy for CKD.

In conclusion, this thesis clarified the molecular pathogenesis of CKD by using murine model for chronic GN, and emphasized the podocyte injuries as an early sign of CKD progression. Importantly, TLR8 was identified as the novel early diagnostic and therapeutic targets for CKD, especially for the podocyte injury in chronic GN. The author strongly believes that these findings lead early diagnosis and novel therapy of CKD in the fields of medicine as well as veterinary medicine.

The original papers of this thesis appeared in *PLOS ONE*, **6**: e16472 (2011), *Am. J. Nephrol.*, **38**: 27–38 (2013), *Genes Immun.*, 15: 182–189 (2014) and *Sci. Rep.*, **4**: 7290 (2014).

Mechanisms of hydrogen sulfide production in the nervous systems

Ryo Miyamoto

Laboratory of Pharmacology, Department of Biomedical Sciences, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060–0818, Japan

In mammals, hydrogen sulfide (H_2S) is produced from L-cysteine through three enzymatic reactions mediated by cystathionine γ-lyase (CSE), cystathionine β-synthase (CBS), and mercaptopyruvate sulfurtransferase (MPST) coupled with cysteine aminotransferase (CAT). In this study, the mechanisms of H_2S production in the rat nervous systems were investigated. Contribution of three enzymatic pathways to H_2S production in the peripheral nervous system (PNS) was first examined, employing dorsal root ganglion (DRG), where cell bodies of sensory

neurons are located, and PC12 cells, which are model cells of peripheral neuron. In PC12 cells and the DRG, CSE was not detected, and expression levels of CBS were low, while cytosolic CAT, mitochondrial CAT, and MPST were all highly expressed. A substantial amount of H_2S was produced from L-cysteine only in the presence of a CAT co-substrate α-ketoglutarate, and the H_2S production was increased by reducing substances, dithiothreitol or dihydrolipoic acid, and was inhibited by a non-selective H_2S-producing enzyme inhibitor (aminooxyacetic acid), CAT competitive substrates (L-aspartate and oxaloacetate) and RNA interference against MPST. MPST was expressed in both the mitochondria and cytosol. H_2S production by CAT/MPST showed bell-shaped pH-dependence, and it was high at pH 8.0 (physiological mitochondrial matrix pH).

In the central nervous system (CNS), CBS is expressed in astrocytes, and is an important source of H_2S. The influence of neurons on CBS expression and its H_2S-producing activity in astrocytes were next evaluated, employing multiple culture systems with different compositions of neurons and astrocytes. H_2S production by CBS was considerably high in neonatal rat spinal cord, but was very low in cultured astrocytes isolated from it. During culture of astrocytes isolated from neonatal rat spinal cord, expression level of glial fibrillary acidic protein (GFAP, astrocyte marker) was increased, while that of protein gene product 9.5 (PGP 9.5, neuronal marker) was markedly reduced, accompanied by the decrease of CBS expression level. In the neonatal rat spinal cord slices, astrocytic, but not neuronal, localization of CBS was shown. In cultured spinal cells isolated from fetal rats, PGP 9.5 was stably expressed, and CBS expression and its H_2S-producing activity were increased as the culture period increased. Impaired CBS expression in neonatal rat astrocytes was restored by co-culture with fetal rat neurons. Treatment with a membrane permeable cAMP analogue dibutyryl cAMP (dbcAMP) restored CBS expression and its H_2S-producing activity to the similar level of that in intact spinal cord. Compared with neonatal rat spinal cord, mRNA level of cultured astrocytes was very low, while that of cultured astrocytes treated with dbcAMP or cultured spinal cells isolated from fetal rats was comparably high.

These results indicate that, in the PNS, H_2S is produced through CAT/MPST, but not through CSE or CBS, and that the CAT/MPST-mediated production of H_2S could be regulated by alteration of mitochondrial conditions. In the CNS, it is demonstrated that astrocytic CBS is an important enzyme for H_2S synthesis, and its expression is controlled by neurons. Neural factors triggering the increase in astrocytic cAMP are suggested to mediate the regulation of CBS expression. This study shows that endogenous H_2S production in the nervous system could be regulated by internal or external factors. H_2S is cytotoxic at high concentration, while, at low concentration, it has also been reported to exhibit antioxidant and anti-inflammatory activities. Changes in production rate of H_2S may be involved in the pathogenesis of neuronal disorders in the PNS and CNS.

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Study of genetic background-dependent diversity of renal failure caused by the tensin2 gene deficiency in the mouse

Hayato Sasaki

Laboratory of Laboratory Animal Science and medicine, Department of Disease Control, School of Veterinary Medicine, Hokkaido University, Sapporo 060–0818, Japan

ICGN mouse is a spontaneous CKD model mouse with null mutation in *Tns2*. Tns2 is a multidomain protein that binds to β-integrin cytoplasmic tails and tyrosine-phosphorylated proteins, and considered to mediate integrin-associated signaling cascades. Despite its ubiquitous expression and predicted function, Tns2-deficiency leads to only alterations of podocytes. Moreover, this pathology critically depends on genetic backgrounds. While the Tns2-deficient podocytes of the resistant murine strains remain intact, the susceptible murine strains with Tns2-deficency, including ICGN mice, develop massive proteinuria, GS and tubulointerstitial fibrosis following the alteration of podocytes foot processes. These evidence suggest a novel podocyte-specific function of Tns2 and the presence of the modifier genes determining the disease phenotype.

In Chapter 1 of this study, backcrossing the resistant B6 mice onto the susceptible ICGN mice, a genome-wide linkage analysis revealed that the resistance to renal failure induced by Tns2-deficiency maps to Chr 2. However, the replacement of Chr 2 in a consomic strain showed that the resistant loci on Chr 2 are insufficient to prevent the alteration of podocyte foot processes due to Tns2-deficiency, suggesting the presence of the other resistant loci outside of the introduced Chr 2 region. In Chapter 2 of this study, firstly, congenic analysis revealed that a FVB strain is susceptible to Tns2-deficiency as well as ICGN and D2 strains. Secondly, no prominent difference was detected in glomerular gene expression between the resistant and susceptible strains. Thirdly, searching for missense variants sorted according to resistant or susceptible strains in podocyte-related genes, *Kif26a*, *Nes*, *Btla* and *Sh3bp1* were identified as the candidate genes associated with $Tns2^{nph}$-induced nephropathy by the prediction tools for functional effects of missense mutation. Each of the candidate genes lies outside of Chr 2 and considered possible to be involved in the formation and maintenance of the unique cytoskeleton of podocyte foot processes.

This study verified that the genetic loci on mouse Chr 2 contribute to the difference in the susceptibility to renal failure induced by Tns2-deficiency, and indicates that the other genetic loci outside of Chr 2 (2.23–88.99 cM) also contribute to the difference in the immediate susceptibility to Tns2-deficiency, and speculates that *Kif26a* is the first candidate gene for the latter contribution and the resistant effects are exhibited by underpinning the skeletal fragility of the podocyte foot process due to Tns2-deficiency. Further study is required to elucidate the podocyte-specific function of Tns2 and identify the resistant genes.

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Studies on *in vitro* production of bovine embryos: effects of penicillamine, hypotaurine and epinephrine on fertilization and individual embryo culture on blastocyst development

Sung-Sik Kang

Laboratory of Theriogenology, Department of Veterinary Clinical Sciences, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060–0818, Japan

Bovine embryo production by in vitro fertilization (IVF) with sex-sorted sperm is an effective method for production of sex-predetermined offspring and it meets demand of dairy and beef industries. If IVF system without optimization of concentration of sperm is developed, the efficiency of in vitro production (IVP) of embryo will be improved. In addition, repeated oocyte collection by transvaginal ultrasound-guided follicular aspiration (Ovum pick-up: OPU) combined with IVP has become alternative to superovulation for embryo production in cattle. However, due to small numbers (about 5 to 6) of immature oocytes can be recovered by OPU, an effective culture method for small numbers of embryos are required.

In chapter I, a mixture of penicillamine, hypotaurine, and epinephrine (PHE) was added to IVF media including theophylline and the effect on fertilization and embryo development were investigated using non-sorted sperm. Further, to estimate sperm quality in the IVF medium with PHE and theophylline, motility and sperm motility parameters were investigated by computer assisted sperm analysis (CASA) system. A combination of PHE and theophylline synergistically accelerates sperm motility and penetration ability. Theophylline activates sperm motility with increasing intracellular cAMP content in sperm and PHE prevents an excessive increase of intracellular cAMP contents in sperm. In addition, when a combination of PHE and theophylline is added to IVF medium at sperm concentration of 2×10^6 cells/ml, relatively high normal fertilization rate (68.2 to 82.4%) from 3 bulls at 18 h after IVF, and high cleavage rate (78.3 to 92.4%) and blastocyst developmental rate (31.9 to 62%) from 9 bulls were achieved. These results indicate that IVF medium with PHE and theophylline at sperm concentration of 2×10^6 cells/ml can produce stably high rate of normal fertilization and blastocyst development using sperm collected from any bull.

In chapter II, to confirm the efficacy of IVF system established in chapter I on IVF using sex-sorted sperm, sperm penetration rates and developmental kinetics of embryos fertilized with sex-sorted sperm in 3 bulls were investigated. Moreover, motility of sex-sorted sperm in supplemented IVF medium was evaluated by CASA system. IVF with PHE mixture and theophylline synergistically enhanced total sperm penetration rate of sex-sorted sperm compared to that with PHE or theophylline only. However, patterns of sperm penetration in sex-sorted sperm of 3 bulls indicate that individual bulls have different penetration abilities and some of them have low pronuclear formation ability compared to non-sorted sperm. Blastocyst development based on the number of cleaved oocytes fertilized with sex-sorted sperm in 3 bulls was low compared to that with non-sorted sperm ($P < 0.05$). Evaluation of sperm quality by CASA revealed that sex-sorted sperm have short longevity compared to non-sorted sperm. The results suggest that an addition of both PHE and theophylline to IVF medium facilitates the penetration to oocytes by sex-sorted sperm.

However, blastocyst development for sorted sperm was inferior to that of non-sorted sperm.

In chapter III, the efficacy of well of the well (WOW) system was investigated when small numbers of embryos were cultured in WOW. Culturing of small numbers of embryos in WOW showed high and stable blastocyst development, and that embryos in WOW did not affected by number of adjacent embryos. Thus, a WOW system can provide high and stable blastocyst development in small group culture wherever embryos are placed in micro-wells of the WOW dish.

In the present study, the author has developed new IVF system which can achieve stable fertilization and blastocyst development using non-sorted sperm from any bull, and has clarified the efficiency of the WOW system to embryo development in individual and small number culture of bovine embryos. However, when sex-sorted sperm was served to the present IVF system, the development to blastocysts was not enhanced although sperm penetration to oocytes was facilitated. It is considered to be necessary to improve the present IVF system for sex-sorted sperm.

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Studies on the virulence factor involved in the pathogenicity in tick-borne encephalitis virus infection

Mizuki Sakai

Laboratory of Public Health, Department of Environmental Veterinary Sciences, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060–0818, Japan

Tick-borne encephalitis virus (TBEV) is maintained among ticks and mammals in nature, and human can be infected with tick bite. Although about 10,000 cases have been reported annually in European countries and Russia, there is no attenuated live vaccine and anti-viral therapy. The virulence of TBEV varies among the strains. However, the mechanism of the viral pathogenicity is unknown. To clarify the mechanism, I tried to identify the virulence factors by reverse genetics, using two different virulence viruses of the Far-Eastern subtype of TBEV; a highly pathogenic strain Sofjin-HO and low pathogenic strain Oshima 5-10. I found that the variable region of the 3'-untranslated region (UTR) is a critical virulence factor and the deletion in the variable region affected the different virulence in mice. Partial deletions or addition of poly A sequence in this region of Oshima also increased the virulence to the same level with Sofjin, although they did not affect the viral multiplication in mice brain and cultured cells. These mutations did not change the production of subgenomic flavivirus RNA from the 3'-UTR, and the induction of interferon (IFN) and IFN-stimulated genes. These data suggested that the whole conformational structure of the variable region is associated with the pathogenicity of the Far-Eastern subtype of TBEV by unknown mechanisms. These findings encourage further research to identify the pathogenic mechanisms of TBEV and develop prevention and therapeutic strategies for TBE.

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Roles of TIM-1 in filovirus entry into cells

Makoto Kuroda

Division of Global Epidemiology, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 001–0020, Japan

Filoviruses including Ebola and Marburg viruses cause rapidly fatal disease characterized by severe hemorrhagic manifestations in humans and nonhuman primates. Consequently, filovirus infections continue to assault human populations, as demonstrated by a recent outbreak in West Africa. Despite extensive research, there are currently no approved vaccines or therapeutics against these viruses. In general, the cellular entry step of viruses is one of the key mechanisms to develop antiviral strategies and the receptor preference is often control susceptibility of host cells to viruses. It has been demonstrated that filoviruses utilize multiple host molecules for entry into cells. However, molecular mechanisms underlying the entry process have not been fully understood, while T-cell immunoglobulin and Mucin domain 1 (TIM-1) and Niemann-Pick C1 (NPC1) are thought to serve as attachment and fusion receptors for filoviruses, respectively.

In chapter I, I demonstrated that TIM-1 was involved in not only filovirus attachment but also subsequent steps of viral entry through the interaction with NPC1 and that their interplay was important for efficient membrane fusion. Moreover, I showed that filovirus infection and viral glycoprotein (GP)-mediated membrane fusion in cultured cells were remarkably suppressed by the treatment with a TIM-1-specific MAb M224/1 that interfered with the interaction between TIM-1 and NPC1. In chapter II, I found that Vero E6 TIM-1 had different primary structures and a greater ability to promote infectivity of vesicular stomatitis viruses (VSVs) pseudotyped with filovirus GPs, compared with TIM-1s derived from the other African green monkey kidney cell lines tested. These results suggest that a polymorphism of the TIM-1 molecules is one of the factors that influence the cell susceptibility to filovirus infection.

The present study provides the first evidence that the interaction between these attachment and fusion receptors is important for filovirus infection, demonstrating an attractive cellular target of antiviral strategies. The detailed structural basis on the molecular interface of the interactions between TIM-1 and NPC1 may provide new insights into the development of antivirals such as low-molecular-weight compounds that can be universally used against filovirus infections. Furthermore, it was also newly suggested that polymorphisms of host molecules might be involved in cell susceptibility to filovirus infection, providing a new insight into the molecular basis for the filovirus host range.

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Characterization of antimicrobial resistant *Escherichia coli* isolated from food producing-animals in Thailand

Kanjana Changkaew

Division of Bioresources, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060–0818, Japan

Antimicrobial use in food-producing animals results in a risk of the evolution of antimicrobial resistant bacteria. The present study has identified high prevalence of multidrug resistance in *E. coli* isolated from shrimp, shrimp environment and pigs. This reflects the selective pressure, caused by intense and less prudent use of the antimicrobials in animal production in Thailand. In addition, commensal *E. coli* can be a reservoir for antimicrobial resistance genes, which can be transferred to pathogenic bacteria. Hence, resistance genes transferring from farm to fork can compromise human health by the potential of producing difficult-to-treat pathogens.

In chapter I, 312 *Escherichia coli* isolates from shrimp farms and markets in Thailand were examined for susceptibility to 10 antimicrobials to survey the risk. The results showed that 17.6% of isolates (55 of 312) were resistant to at least one of the tested drugs, and high resistance rates were observed to tetracycline (14.4%; 45 of 312), ampicillin (8.0%; 25 of 312), and trimethroprim (6.7%; 21 of 312); 29.1% (16 of 55) were multidrug resistant. PCR assay of the *tet*(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E), and *tet*(G) genes detected one or more of these genes in 47 of the 55 resistant isolates. Among these genes, *tet*(A) (69.1%; 38 of 55) was the most common followed by tet(B) (56.4%; 31 of 55) and *tet*(C) (3.6%; 2 of 55). The resistant isolates were further investigated for class 1 integrons. Of the 55 resistant isolates, 16 carried class 1 integrons and 7 carried gene cassettes encoding trimethoprim resistance (*dfrA12* or *dfrA17*) and aminoglycosides resistance (*aadA2* or *aadA5*). Two class 1 integrons, In54 (*dfrA17-aadA5*) and In27 (*dfrA12*-orfF-*aadA2*), were found in four and three isolates, respectively. These results indicate a risk of drug-resistant *E. coli* contamination in shrimp farms and selling places.

In chapter II, cross-sectional study was conducted to investigate antimicrobial susceptibility and extended spectrum beta-lactamase (ESBL)-producing strains and to characterise class 1 integrons in *E. coli* in healthy swine in Thailand. Interestingly, all of the tested isolates (122 isolates) showed drug-resistant phenotypes. High resistance rates were observed to ampicillin (98.4%), chloramphenicol (95.9%), gentamicin (78.7%), streptomycin (77.9%) tetracycline (74.6%) and cefotaxime (72.1%). Fifty-four (44.3%) *E. coli* isolates were confirmed as ESBL-producing strains. Among them, $bla_{\text{CTX-M}}$ (45 isolates) and bla_{TEM} (41 isolates) were detected. Of all $bla_{\text{CTX-M}}$-carried *E. coli*, 37 isolates carried $bla_{\text{CTX-M-1}}$ cluster, 12 isolates carried $bla_{\text{CTX-M-9}}$ cluster and 5 isolates carried both of the clusters. Sequence analysis revealed that $bla_{\text{TEM-1}}$, $bla_{\text{TEM-135}}$ and $bla_{\text{TEM-175}}$ were found in 38, 2 and 1 isolates, respectively. Seventy-one percent (87/122) of the isolates carried class 1 integrons, in which eight distinct drug-resistance gene cassettes with seven different integron profiles were identified in 43 isolates. Gene cassettes were found to be associated with resistance to aminoglycosides (*aadA1, aadA2*, *aadA22* or *aadA23*), trimethoprim (*dfrA5, dfrA12* or *dfrA17*) and lincosamide (*linF*). Genes encoding for beta-lactamases were not found in class 1 integrons. This is the first study to report ESBL-producing *E. coli* and to identify a class 1 integron carrying *linF* gene cassette in swine in Thailand.

It is suggested by these data that the co-selection of multiple drug resistance genes by the usage of some antimicrobials greatly contributes to the wide distribution of MDR bacteria. Therefore, the regulation of antimicrobial use that can select the resistance might be needed in order to control antimicrobial resistant bacteria.

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Epidemiological and bioinformatical analyses of tick-borne pathogens

Yongjin Qiu

Division of Collaboration and Education, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060–0818, Japan

Ticks can transmit a wide range of microorganisms, such as viruses, bacteria, and protozoa, and its distribution is expanding mainly due to climate changes. In addition, emerging tick-borne diseases have recently been increasingly reported worldwide. Therefore, risks caused by ticks and tick-borne diseases are elevating. It is highly suspected that ticks still possess unrevealed pathogens which may threaten human and animal health. In this regard, epidemiological and bioinformatic studies were carried out on tick-borne pathogens, focusing on epidemiology of *Coxiella burnetii*, the causative agent of Q fever in livestock in Zambia and characterization of tick bacterial and viral populations.

In chapter I, *C. burnetii* DNA was detected in Zambian livestock using a PCR assay performed with primers based on a repetitive transposon-like element. Blood samples of cattle and goats were collected in four areas, Monze, Chongwe, Petauke, and Chama. Samples from Chama area in the Eastern Province which is an extensive cattle-raising area showed the highest prevalence of *C. burnetii* DNA in cattle, which agree with the result of a previous serological study of humans showing that samples from Eastern and Western Provinces showed higher positive ratios than in other areas. These results suggested that livestock is one of the risk factors of infection with *C. burnetii* in Zambia.

In chapter II, bacterial flora was analyzed in tick salivary glands by 16S rDNA amplicon analysis with a next generation sequencer. Totally 163 different bacterial genera, including those known as tick-borne pathogens such as *Ehrlichia* and *Rickettsia*, were identified in this study. The principal component analysis revealed that tick bacterial communities in salivary glands had differences in tick species. When compared with a conventional *Rickettsia*-specific PCR assay, this high throughput sequencing approach had higher sensitivity in the detection of rickettsial sequences. Thus, the strategy used in this study makes it feasible to detect both known and as-yet unknown pathogens, and therefore is useful for the surveillance of tick-borne pathogens.

In chapter III, viral community was analyzed in ticks by shot gun sequencing followed by BLASTn and Batch Learning Self-Organizing Map (BLSOM). BLASTn search of the resulting contig data identified 3 viral order and families, including *Mononegaviridales*, *Bunyaviridae*, and

Rhabdviridae. BLSOM is a composition-based data processing method which was designed to separate and cluster sequence fragments based on the similarity of oligonucleotide frequencies without any other taxonomical information. By applying this method, 43 different viral families were found from the same contig data sets used for BLASTn. This approach is useful for the screening of potential viral pathogens without prior knowledge, thus allowing the prediction of the emergence of yet-known tick-borne diseases. Both experimental and epidemiological studies are necessary to assess the ricks of these viruses for human and animal health.

The findings obtained from this study can provide valuable basic information for the prediction of and our preparedness against emerging tick-borne diseases.

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