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INFORMATION

Hokkaido University conferred the degree of Doctor of Philosophy on September 25, 2013 to 6 recipients.
The titles of theses and other information are as follows:

A new class of endoplasmic reticulum export signal ΦXΦXΦ for transmembrane proteins and its selective interaction with Sec24C

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Protein export from the endoplasmic reticulum (ER) depends on the interaction between a signal motif on the cargo and a cargo-recognition site on the coatomer protein complex II (COPII). A hydrophobic sequence in the N-terminus of the bovine AE1 anion exchanger facilitated the ER export of human AE1Δ11, an ER-retained AE1 mutant, through interaction with a specific Sec24 isoform. The cell surface expression and *N*-glycan processing of various substitution mutants or chimeras of human and bovine AE1 proteins and their Δ11 mutants in HEK293 cells were examined. The N-terminal sequence (V/L/F)X(I/L)X(M/L), ^{26}VSIPM^{30} in bovine AE1, which is comparable with ΦXΦXΦ, acted as the ER export signal for AE1 and AE1Δ11 (Φ is a hydrophobic amino acid and X is any amino acid). The AE1-Ly49E chimeric protein possessing the ΦXΦXΦ motif exhibited effective cell surface expression and *N*-glycan maturation via the COPII pathway, whereas a chimera lacking this motif was retained in the ER. A synthetic polypeptide containing the N-terminus of bovine AE1 bound the Sec23A/Sec24C complex through a selective interaction with Sec24C. Co-transfection of Sec24C-AAA, in which the residues ^{895}LIL^{897} (the binding site for another ER export signal motif IXM on Sec24C and Sec24D) were mutated to ^{895}AAA^{897} specifically increased ER retention of the AE1-Ly49E chimera. These findings demonstrate that the ΦXΦXΦ sequence functions as a novel signal motif for the ER export of cargo proteins through an exclusive interaction with Sec24C.

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Characterization and interspecies diversity of xenobiotic metabolism: a study of phase I oxidation and phase II conjugation reactions

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The species differences with regards to the capacity to metabolize and eliminate drugs and other xenobiotics from the body are typically substantial, complicating the effective use of drugs, as well as minimizing the ability to predict the adverse consequences of xenobiotics. The key factor to determine the species differences are the xenobiotic metabolizing enzymes. It has been reported that the xenobiotic metabolism is divided into phase-I and II reactions. In phase-I reaction, the main enzymes are the cytochromes P450 (CYP). Phase-II enzymes also play an important role in the metabolism of phase-I metabolites to more water-soluble forms. In particularly mammals, glucuronidation and sulfation extremely contribute to metabolisms of various xenobiotics. In the present study, I aim to evaluate the interspecies differences of both phase-I and phase-II reaction by using warfarin and pyrene as a model compounds.

Warfarin, the anticoagulant drug, was used as a model to study the characterization of phase-I, CYP and non-CYP reaction, in rats and chickens. I found that the metabolic activity of warfarin was drastically higher in chicken microsomes and cytosol fractions than that of rats. Also I found that there was the interspecies difference in the stereoselectivity of warfarin between rats and chickens.

To estimate the interspecies differences of phase-II reaction from various mammalian species, I constructed the method to analyze urinary metabolites of pyrene that is one of the typical polycyclic aromatic hydrocarbons (PAHs). Based on the constructed method, urinary pyrene metabolites from 16 mammalian species with non-experimentally exposed condition were analyzed. The results indicated that glucuronide conjugations were mainly eliminated via urine in various mammals, except cats and ferrets. Interestingly, sulfate conjugate was detected in pig urine, although pig is well known species that have low aryl-sulfotransferase (SULT) activity. Based on the kinetic analysis, high V_{max}/K_m of SULT was found in pig, which is higher than that of rats.

To summarize the study, I constructed the novel method to characterize interspecies differences, and clear interspecies differences of xenobiotics metabolism of both phase-I and phase-II were observed.

The original paper of this thesis appeared in *Poult. Sci.*, **90**: 2775–2781 (2011).

Molecular investigation of tick-borne protozoan parasites at the livestock-wildlife interface in Kenya and evaluation of a candidate anti-tick vaccine antigen

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Vector-borne diseases are a major hindrance to economic development in Africa. As obligate haematophagous feeders, ticks transmit a wide range of microorganisms, including many that are pathogenic to humans, livestock and wildlife. In Kenya, as in many parts of Africa, livestock-keeping is often practised in areas with abundant wildlife. This creates geographical zones (livestock-wildlife interfaces) supportive of disease transmission between domestic animals and wildlife. Understanding pathogen occurrence and identity in these areas, and evaluating vaccine efficacy of ferritin2 (FER2), a tick gut protein, was the focus of the present studies.

In chapter 1, *Babesia* and *Theileria* occurring in wild felids from Kenya were identified through polymerase chain reaction-reverse line blot (PCR-RLB). A simple and versatile method, RLB enables simultaneous detection and differentiation of multiple pathogens from a host blood. Multiple probes on the membrane enable detection of mixed infections. In combination with sequencing of DNA loci that are highly conserved in genus, for instance, RLB can help reveal the occurrence of novel parasites. Presently, genetic variants of *Babesia canis* were found in blood from a pair of lions with a history of suspected mineral deficiency. This finding is significant since fatal babesiosis linked to co-infection with canine distemper virus has been reported in lions recently in East Africa. From the leopard specimens, *Babesia leo (B. leo)* was detected by RLB; however, the limited number of 18S rRNA sequences obtained from plasmid clones did not match published sequences of *B. leo,* perhaps because the sequencing was of low quality. *Theileria*-like parasite of unknown pathogenicity was detected by RLB in the cheetah samples, and 18S rRNA gene sequencing confirmed it was a true *Theileria* species.

In chapter 2, two oligonucleotide probes specific to *Theileria* of giraffes were designed and evaluated. Alongside 18S DNA sequencing, the new probes indicated that at least two populations of *Theileria* occur in the giraffes from Kenya, with multiple genetic variants within them. High sequence homology with genotypes reported to cause fatalities in South African giraffes suggests that the Kenyan isolates too maybe pathogenic.

In chapter 3, the question of whether the waterbuck, a bovidae, is host to parasites infective to cattle was investigated. In 26 waterbuck blood samples from malura, a *Theileria parva (T. parva)* endemic area, there was no evidence of *T. parva* infection by both nested PCR and RLB specific to this parasite. Cattle blood specimens from the same locality were however infected with numerous *Theileria* spp., including *T. parva* and *Theileria* sp. (buffalo) (67.4%), the causative agents of East Coast fever and Corridor disease, respectively. RLB and 18S phylogeny of the waterbuck parasites indicated that three distinct species maybe present in these animals, including one clade that is highly similar to *Theileria equi*, a known pathogen of equines.

FER2 has been shown to be critical for iron homeostasis in ticks, blood feeding and

reproduction, and therefore suitable as a component for anti-tick vaccines. In chapter 4, vaccine efficacy of FER2 was evaluated in a heterologous challenge involving *Ixodes persulcatus (I. persulcatus)* and *Ixodes ovatus (I. ovatus)* ticks from Hokkaido, Japan. Cloning of FER2 showed high amino acid conservation, consistent with past studies. Vaccination with *I. persulcatus* recombinant FER2 (I.pFER2) elicited a strong antibody response in experiment animals. *I. persulcatus* and *I. ovatus* ticks feeding on I.pFER2-vaccinated animals had 45–55% weight reduction compared to the controls. Furthermore, oviposition in *I. persulcatus* ticks was clearly reduced, both in the number of eggs and their appearance, consistent with past findings. A FER2 homologue from the African tick *Rhipicephalus appendiculatus* was also identified in the present study for future field evaluation in Africa.

The original papers of this thesis appeared in *Acta Trop.*, **124**: 71–78 (2012) and *Parasitol. Int.*, **62**: 448–453 (2013).

Study on the involvement of maternal immune response in the natural occurrence of persistent infection with bovine viral diarrhea virus

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Bovine viral diarrhea virus (BVDV) infection has a significant impact on both dairy and beef cattle producers worldwide. Animals persistently infected (PI) with BVDV serve as a continuous source of the virus due to life-long shedding. Early detection and elimination of PI animals are important for the control of BVDV. In addition, the prevention of PI animal production should be considered. In order to prevent PI animal production, the mechanism of persistent infection should be thoroughly investigated. Currently, there is no examination method to detect cows carrying PI fetuses under field conditions prior to birth of the PI calf. It was reported in experimental fetal infections that the maternal immune response has been suspected as closely concerned to the PI calf production. Trials for the detection of dams carrying PI fetuses using the maternal immune response were performed using experimental fetal infection. For the control of BVDV infection, more detailed analyses of the maternal immune response under the natural occurrence of PI calves should be estimated. Thus, the aim of this thesis was to investigate involvement of maternal immune response in the natural occurrence of persistent infection with BVDV and analyze the possible mechanism of PI calf production.

In chapter 1, a dairy herd including 50 milking cows and 40 heifers and calves was selected from natural cases of BVDV-contaminated herds. This herd was detected with high prevalence of BVDV PI calves. Nine PI animals including a milking cow and 8 newborn calves were detected in the herd within 4 months. Prevalence of PI animals in this herd was estimated 7.0%, which was very high compared to that estimated in previous reports. All

newborn PI calves were strongly suspected to have a single origin of infection as estimated from the homology of the virus genes. Moreover, all PI calves were produced from non PI dams. Therefore, this herd was a worthy case to study production of PI calves in a natural occurrence.

In chapter 2, in order to estimate the maternal immune response to the natural occurrence of PI calves, the high prevalence herd investigated in chapter 1 was used as an experimental herd. C-X-C chemokine receptor type 4 (CXCR4) expression and cytokine expressions including interleukin-4 (IL-4), IL-6, IL-10, IL-12p40, interferon-α (IFN-α), IFN-γ and transforming growth factor-β (TGF-β) in the cows of this herd were investigated. There were no significant differences in CXCR4 and cytokine expressions between the dams of PI calves and the dams of non PI calves in the herd. In the comparison among the herds, CXCR4 expressions in the PI-producing herds were significantly lower than the BVDV-free herd. The level of CXCR4 expression in the high prevalence herd was similar to that in the low prevalence herd. Based on the cytokine profiles, the high prevalence herd and the BVDV-free herd had almost same immunological responses. IL-10 was significantly higher in the high prevalence herd and the BVDV-free herd than the low prevalence herd.

In this thesis, maternal CXCR4 and cytokine expressions were investigated in the dams of the herd with high prevalence of PI calves. CXCR4 expressions in the high prevalence herd showed no significant differences between dams of PI calves and non PI calves. These findings indicated that both dams had an equal risk for PI production inspite of the production of non PI calves. CXCR4 expressions in both of the dams in this herd were significantly lower than the BVDV-free herd.

It might be possible to predict the susceptibility of the herd to the transplacental persistent infection based on the maternal immune response. The combination of low expression of CXCR4 and high expression of IL-10 might be closely concerned with some bias for the production of PI calves.

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Molecular epidemiological study of selected infectious diseases of livestock in Zambia

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This study showed that molecular tools are useful in the studies on the epidemiology and basic biology of infectious diseases of livestock in the field. The tools employed in this study both produced entirely new information hence proving the importance of such tools in the study of infectious diseases of livestock.

In the chapter I, cattle blood samples from Isoka and Petauke districts of Zambia were screened for *T. parva* DNA. A cohort of *T. parva* positive samples were analyzed using a panel of 9 microsatellite markers. Significant differentiation between the Isoka and Petauke populations was observed. Linkage disequilibrium was also observed when Isoka and Petauke district were treated as a single population. Separate analysis

produced linkage disequilibrium in Kanyelele and Kalembe areas in Isoka district, Isoka district overall and Petauke district. As such, the study on population genetics of *T. parva* from Isoka and Petauke districts showed a low level of genotype exchange between the districts, a high level of gene diversity within each district population, absence of panmixia and consequently, genetic and geographic sub-structuring between the districts. A higher multiplicity of infection was observed in Petauke as compared to Isoka district based on the average number of alleles identified through the use of capillary electrophoresis. With respect to this, theileriosis control strategies based on the use of cocktail vaccines in either Northern or Eastern provinces should be delicately carried out so as to prevent the introduction of new genotypes in naïve areas.

In chapter II, the lineage of rabies virus (RABV) in Zambia, using phylogenetic analysis of the nucleoprotein gene was determined using samples collected over an 11 year period. The level of genetic diversity of RABV strains in different hosts was also determined using the phylogenetic analysis of the glycoprotein gene. Analysis of the *N gene* produced two phylogenetic clusters belonging to the Africa 1b lineage present in eastern and southern Africa. One cluster comprised exclusively of Zambian strains. The other was heterogeneous and included strains from Tanzania, and Mozambique. Analysis of the *G gene* showed the presence of similar RABV strains in different hosts and regions of Zambia. I also designed primers for RT-LAMP assay using the consensus sequence of the *N gene*. I then confirmed the specificity and reproducibility of the RT-LAMP assay using actual clinical specimens. The RT-LAMP assay proved to be useful for routine diagnosis of rabies in Zambia. In this study, I established that the dog is the most probable source of infection to livestock and human beings and as such vaccinating these dogs will prevent spread of rabies to livestock and thus preventing loss of livestock.

The molecular tools used in this study proved to be a valuable way of obtaining epidemiological data on the infectious diseases under study. These tools can also be applied to other pathogens, which will greatly enhance the prevention of the spread of diseases. The prevention of the spread of these diseases will facilitate the improvement of livestock and consequently, the livelihoods of the general population. Further research, however is needed so that cost effective disease control strategies, based on concrete knowledge of their epidemiology should be designed and implemented.

The original papers of this thesis appeared in *Parasit. Vectors*, **5**: 225 (2012) and *Virus Res.*, **163**: 160–168 (2012).

Ecological and molecular epidemiological studies of Japanese encephalitis virus and Culex flavivirus in Toyama Prefecture

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Japanese encephalitis virus (JEV) and Culex flavivirus (CxFV) belong to the genus *Flavivirus* within the family *Flaviviridae*. JEV exists in an enzootic cycle between mosquitoes and vertebrate hosts such as pigs and birds. JEV is distributed in East and South Asia, and causes severe encephalitis in humans. CxFV is an insect-specific flavivirus and genetically related with the other insect flaviviruses.

The author isolated 87 JEV strains from mosquitoes and pigs in Toyama Prefecture during 2005–2009. The result suggests JEV still circulates between mosquitoes and pigs in Toyama Prefecture, and circulation of JEV is correlated with the prevalence of mosquitoes. According to the nucleotide sequence of the envelope and capsid/premembrane genes, all isolates belong to genotype I. These isolates were divided into the major type and the two minor types. The major type of JEV might have remained in Toyama Prefecture and gradually changed over five years, while two minor types of JEV might have migrated from other countries and then become extinct. All isolates collected in 2008 and 2009 had a novel deletion in the 3' untranslated regions. Furthermore, the author reports that the peak level of JEV circulation occurs later in the year than in the past. This might be related to the recent decrease in the prevalence of Japanese encephalitis in Japan, in addition to the effects of human vaccinations. JEV strains circulating in areas of the temperate zone may consist of locally maintained viruses and the viruses migrated from other areas.

The author isolated and genetically characterized CxFV strains from *Culex tritaeniorhynchus* and *Culex pipiens* group mosquitoes. The minimum infection rate of CxFV within *Cx. tritaeniorhynchus* populations was much lower than that within *Cx. pipiens* group. The complete genome sequences of 11 CxFV isolates and five reference strains had 95.2–99.2% nucleotide and 98.1–99.8% amino acid identities. Phylogenetic analysis showed that the 11 isolates were divided into four clusters. One cluster consisted of five isolates from *Cx. pipiens* group and *Cx. tritaeniorhynchus* from one site and their nucleotide sequences almost completely matched. An isolate had a unique sequence, suggesting that it was introduced from abroad. CxFV strains were divided into several groups according to countries when nucleotide sequences of CxFV available in GenBank and 11 Toyama isolates were compared. These results suggest that CxFV is maintained in nature among *Culex* mosquitoes in a mosquito habitat-specific but not a species-specific manner.

The original papers of this thesis appeared in *Am. J. Trop. Med. Hyg.*, **84**: 695–708 (2011) and *J. Med. Entomol.*, **50**: 300–309 (2013).