



# HOKKAIDO UNIVERSITY

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The title of theses and other information are as follows:

## **Morphological and genetic analyses on the cystic rete ovarii in MRL/MpJ mice**

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In mammalian species, ovarian cysts are a major cause of female reproductive dysfunction and derived from several sources, including ovarian surface epithelium, follicles, corpus luteum, and rete ovarii. In veterinary medicine, infertility or subfertility in farm animals due to ovarian dysfunction is important problem, but fundamental cause of ovarian cysts are not yet clarified. Meanwhile, MRL/MpJ (MRL) mice are model for autoimmune diseases, and show ovarian cysts derived from rete ovarii in high frequency. The rete ovarii, which are convoluted tubules lined by ciliated or nonciliated epithelium, is known as mesonephric tubules in embryogenesis and persist in adult female throughout the whole-life. Even its function in adult has not yet been elucidated, ovarian cysts from rete ovarii are common in many species such as cow, swine, feline, canine and rodent. In MRL mice, because of useful model for ovarian cysts derived from rete ovarii, it is important to elucidate the morphological dynamics and the genetic background by investigating sequential whole-mount observation and quantitative trait loci (QTL) analysis.

In the chapter 1, the rete ovarii in C57BL/6 (B6) mice consisted of a series of tubules including the extraovarian rete (ER), the connecting rete (CR), and the intraovarian rete (IR) based on their location. Although the ER in B6 mice was

composed of highly convoluted tubules lined by both ciliated and non-ciliated epithelium, the tubules in the CR and the IR had only non-ciliated cells. In MRL mice, dilations of the rete ovarii initiated from the IR rather than the ER or the CR. Although the cellular types lining the lumen of the rete ovarii were the same as those in B6 mice, MRL mice showed a variety of ER morphology. In particular, the connections between ER and ovary tended to disappear with increasing age and the development of ovarian cysts. Furthermore, the epithelium lining large ovarian cysts in MRL mice had ciliated cells forming the cluster. Based on these findings, it was suggested that cystic changes of the rete ovarii in MRL mice were caused by the dilations of the IR with invasion of the ER and the CR into the ovarian medulla. These data provide new pathological mechanisms for cystic rete ovarii formation.

In the chapter 2, to elucidate the genetic background during development of ovarian cysts, the author performed QTL analysis using 120 microsatellite markers which covered the whole genome of murine chromosomes (Chrs) and 213 backcross progenies between MRL and B6 mice. The quantitative trait was measured as the circumferences of rete ovarii or ovarian cysts. As a result, suggestive linkages were detected on Chrs 3, 4, 6, and 11, and significant linkage

appeared on Chr 14 by interval mapping. The author thereby designated the 33.21–37.26 cM region of Chr 14 “MRL Rete Ovarian Cysts (*mroc*)”. The peak regions of Chrs 4 and 14 in particular showed close additive interaction ( $P < 0.00001$ ). From these results, the author concluded that multiple loci on Chrs 3, 4, 6, 11, and 14 interact themselves to result in development of cystic rete ovarii in MRL mice.

In the chapter 3, a significant linkage appeared on Chr 6 at the marker position *D6Mit188* (likelihood ratio statistic (LRS) = 18.5) in the analysis of 113 F2 intercross progenies. From the results, a locus on Chr 6 was identified as *mroc2*,

the second major locus associated with ovarian cyst formation in MRL mice.

In conclusion, it is noted that cystic rete ovarii in MRL mice result from the recruitment of CR and ER epithelium, not active proliferation of epithelia. Furthermore, we concluded that development of cystic rete ovarii in MRL mice is a multifactorial disorder, affected by several loci directly or indirectly, especially the *mroc* and the *mroc2*. The information from this unique phenotype in a typical autoimmune model would be useful to unveil various etiopathological factors of cystic rete ovarii in animals and human.

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## Specificity and function of filovirus glycoprotein-specific antibodies

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Ebola virus (EBOV) and Marburg virus (MARV) belong to the family *Filoviridae* and are classified as biosafety level 4 agents. While MARV consists of a single species, five distinct EBOV species are known. Since outbreaks of Ebola and Marburg hemorrhagic fever in central Africa, associated with high lethality and serious social consequences, are occurring frequently in the past decade, EBOV and MARV have become major public health priority. Preparation of valuable diagnostic methods and understanding of the viral pathogenicity are required to control EBOV and MARV infection.

Because of the lack of species-specificity of diagnostic methods, previous serological surveys have not illustrated the natural distribution of each filovirus species. In this regard, in Chapter

I, I established an enzyme-linked immunosorbent assay using the recombinant viral glycoproteins (GPs) as antigens, which enable us to detect species-specific IgG and IgM antibodies with high sensitivity. This assay would be helpful for retrospective seroepidemiologic surveys aimed at detecting species-specific antibody responses.

Antibody-dependent enhancement (ADE) of infection has been considered to be a significant obstacle to the development of effective vaccines for some viruses, since virus-specific antibodies conferred by vaccination potentially facilitate the entry of the virus into target cells, sometimes resulting in increased severity of the disease. In Chapter II, it was demonstrated that some antibodies to MARV GP were exploited for the efficient virus entry leading to increased

infectivity in vitro. The results correlated the distinct capacity of GP to induce enhancing antibody with the difference in pathogenicity between MARV strains, leading to a hypothesis that ADE may have an impact on the severity of symptoms of MARV infection.

The present study provides a useful method for diagnosis and epidemiological study of filovirus infection and raises an important issue to be considered in development of vaccines and passive prophylaxis with antiviral antibodies toward the control of MARV infection.

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## Characterization of influenza A viruses isolated from wild waterfowl in Zambia

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Influenza A viruses are zoonotic pathogens of global importance. The virions possess a host-derived lipid membrane which harbours the surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA). Based on the antigenic properties of HA and NA, influenza A viruses are classified into subtypes. Each of the currently known subtypes [i.e. 16 HA (H1-H16) and 9 NA (N1-N9)] and in many possible combinations has been identified in wild waterfowl and shorebirds that are recognized as the major natural reservoir of avian influenza viruses (AIVs).

Since the emergence of the H5N1 highly pathogenic avian AIV in Asia and its eventual global spread, together with the understanding that all mammalian influenza A viruses, including pandemic influenza strains have their origin in the AIV pool, great emphasis has been directed towards AIV surveillance worldwide with the aim of averting the threat of influenza pandemics. However, the African continent is highly underrepresented in influenza surveillance. In the present thesis, valuable data for a better understanding of the ecology and epidemiology of AIVs in Zambia has been provided.

During active AIV surveillance conducted in Lochinvar National Park in Zambia in 2006–2009, 13 nonpathogenic strains of various subtypes (H3N6, H3N8, H4N6, H6N2, H9N1, and H11N9) were isolated from wild waterfowl. In chapter 1, it was demonstrated that all the gene segments of the first AIV isolate (H3N6) in Zambia belonged to the Eurasian lineage and that some genes may have been derived from Europe, Asia and Africa through reassortment events facilitated by the overlap of flyways across these regions.

This finding was reiterated in chapter II where twelve more isolates were genetically analyzed. It was notable that some genes were closely related to those of AIVs isolated from domestic birds (e.g. ostrich) in South Africa, suggesting possible AIV exchange between wild birds and poultry in southern Africa. I showed in chapter II that several AIV subtypes circulate in Zambia and that they could persist in a southern Africa ecosystem at times when Eurasian migratory birds are usually absent or rare. I further demonstrated that some AIVs isolated from wild waterfowl in Zambia may have the

potential to infect mammals directly without adaptation. It is hoped that continued monitoring of AIVs in wild and domestic birds in southern Africa and the complete characterization of

isolates may help in averting the sustained threat of influenza-related disasters in animals as well as in humans.

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The original papers of this thesis appeared in *Arch. Virol.*, **154**: 1517–1522 (2009) and *J. Gen. Virol.*, **92**: 1416–1427 (2011).

## Effects of chondroitin sulfate-E on Japanese encephalitis virus infection

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Glycosaminoglycans (GAGs) are components of proteoglycans and are classified according to their sugar backbone structure and sulfation pattern, which can vary in both quantity and positions substituted by sugar residues and/or sulfate groups. Different GAGs have diverse functions in the body. In this study, the possible role of E-disaccharide units of chondroitin sulfate (CS), a GAG involved in neuritogenesis and neuronal migration, was evaluated in Japanese encephalitis virus (JEV) infection. Soluble CS-E derived from squid cartilage inhibited JEV infection in non-neural cell lines, i.e. Vero cells and BHK-21 cells, by interfering with viral attachment. In contrast, sCS-E enhanced viral infection in the mouse neuroblastoma cell line Neuro-2a, despite the fact that viral attachment to Neuro-2a cells was inhibited by sCS-E. This enhancement effect seemed to be related to increased viral RNA replication due to the inhibition of JEV-induced interferon  $\beta$  (IFN- $\beta$ ) synthesis by sCS-E. In Neuro-2a cells, knockdown of N-acetyl-D-galactosamine 4-sulfate 6-O-sulfotransferase, which synthesizes E-disaccharide units from precursor A-disaccharide units in CS chains, increased the level of

constitutive IFN- $\beta$  expression and reduced susceptibility to JEV infection. The amount of E-disaccharide units was larger in the brains of 4-day-old rats, which are highly susceptible to JEV infection, than in those of 17-day-old JEV-resistant rats. Intracerebral co-administration of sCS-E with JEV in 17-day-old rats resulted in higher brain viral loads relative to rats infected without sCS-E administration.

Taken together, these results showed the paradoxical effects of sCS-E on JEV infection in different cell types. In neural cells, which are major target of JEV, E-disaccharide units enhanced JEV infection through the reduction of IFN- $\beta$  expression. Moreover, this enhancement effect was also observed in a rat infection model intracerebrally inoculated with JEV and sCS-E and virus. In conclusion, this study suggests that E-disaccharide units, which are significant in the CS chains from the developing brain, is a candidate host factor involved in the age-dependent susceptibility of neurons to JEV. These results provide a model for understanding the role of E-disaccharide-containing CS chains in viral infection in central nervous system.

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The original paper of this thesis appeared in *Biochem. Biophys. Res. Commun.*, **409**: 717–722 (2011).

## **Molecular epidemiology of *Theileria orientalis* in Australia and development of a molecular method for differentiation of *T. orientalis* genotypes**

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Bovine theileriosis caused by *T. orientalis* is prevalent throughout Japan, Korea and parts of Russia and China. The distribution of benign *Theileria* related to *T. orientalis* often referred to as *T. buffeli* appears to be worldwide, but is less frequently associated with clinical disease although some fatal cases have been reported to occur in America. Based on the major piroplasm surface protein (MPSP) gene, there are eight genetic types (genotypes) currently comprising the *T. orientalis* complex. These types include type1 (Chitose), type2 (Ikeda), type3 (Buffeli) and type-4-8 yet to be classified with taxonomically. Mixed infection with different types of *T. orientalis* in cattle are common in Japan, Korea, and China. Herein, I report the molecular epidemiology of *T. orientalis* in Australia and development of molecular diagnostic method for differentiation of *T. orientalis* genotypes.

In recent years, theileriosis has been suspected of being responsible for a number of outbreaks of disease in cattle near the coast of New South Wales Australia. Therefore, in the first part of my study, I identified and characterized the species of *Theileria* in cattle on six farms in New South Wales where disease outbreaks had occurred, and compared this with *Theileria* from three disease-free farms in Queensland that is endemic for *Theileria*. Special reference was made to sub-typing of *T. orientalis* by type-

specific PCR and sequencing of the small subunit (SSU) rRNA gene, and sequence analysis of the gene encoding a polymorphic merozoite/piroplasm surface protein (MPSP). Nucleotide sequencing of SSU rRNA and MPSP genes revealed the presence of four *Theileria* MPSP and SSU rRNA genotypes: Buffeli, Ikeda, Chitose and MPSP type 4, or SSU rRNA type C. The majority of animals showed mixed infections while a few showed single infection.

Noting that the current detection and identification methods for the *T. orientalis* genotypes have got limitations as mentioned above, I developed a novel easy to use sensitive and specific diagnostic method for population structure analysis of *T. orientalis* types specifically Ikeda, Chitose and Buffeli the most frequently encountered genotypes. By applying ITS1 and ITS2 spacers in fragment genotyping, I utilized primers flanking the two ribosomal RNA internal transcribed spacers (ITS1 and ITS2). Due to varying degrees of sequence polymorphism in the ITS regions found within and between species, I exploited the insertions and or deletions in these regions which resulted in different fragment sizes. On the basis of fragment size polymorphism, it was able to discriminate the three commonly found types of *T. orientalis*. ITS1 was capable of discriminating all three types (Ikeda;251 bp, Chitose;274 bp and Buffeli;269 bp)

in one single reaction by fragment genotyping. However, using ITS2, Ikeda (133 bp) a more pathogenic type was distinguishable from Buffeli/Chitose (139 bp). In addition, parasite load was quantified in experimental animals using ITS1. When compared with previous PCR detection method, ITS-based genotyping was found to be more sensitive methods with high specificity in

population analysis and can be deployed in molecular epidemiology studies.

In conclusion, this study provides a new insight into molecular epidemiology of *T. orientalis* especially in the Oceanian regions, and a new tool of sensitive diagnosis and parasite population analysis.

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The original papers of this thesis appeared in *Parasites Vectors*, **4**: 22 (2011) and *Infect. Genet. Evol.*, **11**: 407–414 (2011).

## Molecular epidemiological studies of protozoan diseases in domestic animals in the Sudan

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This work involved the molecular epidemiological aspects of two common and economically important protozoan diseases in Sudan; trypanosomiasis and equine piroplasmosis.

In the first part, characterization of Sudanese isolates of *Theileria equi*, and *Babesia caballi*, the causative agents of equine piroplasmosis was carried out. A number of 127 and 509 blood samples were collected in 2007 and 2010, respectively, and the small subunit rRNA gene was amplified, sequenced and analyzed to reveal possible strain differences and the presence of novel genotypes. Eleven Sudanese isolates of *T. equi* were found to be genetically distinct from all previously known isolates and were considered novel genotypes. The analysis also revealed that three Sudanese isolates shared some similarities with *T. equi* found in other countries including Spain and South Africa. On the other hand the prevalence of both *T. equi* and *B. caballi* was estimated to be 36% in the samples collected in 2010.

The second part reported an outbreak of

bovine trypanosomiasis in the Blue Nile State, Sudan that involved the infection with four *Trypanosoma* species in cattle. The outbreak occurred in 2010 in indigenous Kenana and Fulani cattle breeds. A total of 210 blood samples from cattle and a few from other domestic animals were collected and examined by using conventional parasitological techniques such as the hematocrit centrifugation techniques and microscopic examination of Giemsa-stained thin blood films. They were also tested by polymerase chain reaction (PCR) targeting the internal transcribed spacer region 1 (ITS1) which provides a multi-species-specific diagnosis. Parasitological examinations revealed that 33.3% of the animals were infected with *T. vivax* and 10% with *T. congolense*. ITS1-PCR, however, detected the presence of four *Trypanosoma* species namely *T. vivax*, *T. congolense*, *T. simiae* and *T. brucei* in 56.7% of the investigated animals. None of the samples was shown positive for *T. brucei rhodesiense*. The identification of *T. simiae* was further confirmed by sequencing and phylogenetic

analysis. It was hypothesized that variant parasite genotype(s) have been introduced to Sudanese cattle from Ethiopia, a tsetse belt region.

The third part represented a cross-sectional investigation carried out in 2009 on 687 samples from camels representing geographically distinct zones in the Sudan to detect all possible trypanosoma species infective to camels. The prevalence rate varied by region ranging from 7.1 to 57.1 %. Using generic ITS1-PCR, it was shown that all positive camels were infected with a single parasite species; *T. evansi*. Additionally, the absence of RoTat 1.2 gene that is reported to be *T. evansi* specific was demonstrated in thirteen out of thirty *T. evansi*-positive samples. Different prevalence rates of camel trypanosomiasis were shown for different geographical locations. It was concluded that camel trypanosomiasis is highly prevalent, which strengthens the need

solid control measures.

The fourth part investigated genetic variation at 15 microsatellite loci of *T. evansi* isolated from camels in Sudan and Kenya to evaluate the genetic information partitioned within and between individuals and between sites. A strong signal of isolation by distance was detected across the area sampled. The results also indicated that *T. evansi* is, most likely, purely clonal and structured in small units at very local scales. In addition there are numerous allelic drop out in the data. This may be explained if the parasite often sexually recombines without the need of tsetse fly as the definitive host, or the recurrent immigration from sexually recombined *T. b. brucei* had occurred.

In conclusion, the present studies based on advanced molecular technologies may clue in to novel approaches to control protozoan diseases which constrain animal production in Sudan.

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The original papers of this thesis appeared in *Parasitol. Res.*, **106**: 493–498, (2010), *Parasites Vectors*, **4**: 31, (2011), *Parasites Vectors*, **4**: 74, (2011) and *PLoS Neglect. Trop. D.*, **5**, e1196, (2011).