北海道大学グローバルCOEプログラム

Global COE Program
Progress Report FY 2010
Presentation

2010年度 事業推進担当者研究成果発表会

Programme & Abstract

■場所：北大 獣医学研究科　講堂
■日時：　2011年3月1日(火)10:00～16:30

Venue: Conference Hall
Graduate School of Veterinary Medicine, Hokkaido University
Time & Date: 10:00～16:30, March 1 (tue), 2011

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■Cultivation of Human Resource Group■ Chairperson: Chihiro Sugimoto

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Mutsumi Inaba

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Yasuhiko Suzuki

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Tadaaki Miyazaki

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Closing Speech: Hiroshi Kida, Leader of Global COE Program

Urgent motion for the prompt eradication of H5N1 highly pathogenic avian influenza virus from Asia

Hiroshi Kida, DVM, PhD, MJA
Program Leader, Professor, School of Veterinary Medicine,
Director, Research Center for Zoonosis Control, Hokkaido University
Head, OIE World Reference Laboratory for Avian Influenza
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Since late 2003, H5N1 highly pathogenic avian influenza virus (HPAIV) has seriously affected poultry in Eurasia and Africa. The H5N1 HPAIV has persisted over several years in many areas and as of 2011, four countries; China, Vietnam, Indonesia, and Egypt are endemically infected. Well over a billion of birds have died from infection or been killed for the control purposes. A HPAIV is generated when a nonpathogenic virus brought in by migratory birds from their nesting lakes in the north is transmitted to chickens via domestic ducks, geese, quails, turkeys, etc. and acquires pathogenicity for chickens with repeated multiple infections in the chicken population. It was confirmed that 520 people have been infected with the H5N1 virus, 307 of whom died in Asia, the Middle East and Africa since 2004 (as of 9 February 2011). Sum of the numbers of people infected with the H5N1 HPAIV in the four countries is more than 86 % of total cases in 15 countries.

The reason why the H5N1 HPAIV strains have persisted in the world for more than 8 years and antigenic variant viruses been selected is that the above 4 countries have been using vaccine instead of stamping out policy which is the golden standard for the control of HPAIV infection. As a result, the HPAIV returned to migratory water birds from domestic poultry during over-wintering and many feral water birds die on the way back to their northern territory in Siberia in April and May. Some water birds must have brought HPAIVs to their nesting lakes in Siberia. At last 14 October 2010, we isolated HPAIVs from fecal samples of ducks flying from Siberia to Lake Ohnuma in Wakkanai, Hokkaido, Japan. We, thus, immediately warned to strengthen surveillance of avian influenza in wild birds and poultry. Then outbreaks of HPAIV infection have occurred in 14 chicken farms and wild birds in many places in Japan. It has been reported that Korea is in much worse situation.

Then, how should the HPAIV infection be controlled in poultry?

Stamping-out without misuse of vaccine is only way, so far. Otherwise, this disaster must occur every year in over Asian countries.

Thus, we here urgently propose to eradicate the H5N1 HPAIV from Asia by international collaboration.

Surveillance of human African trypanosomiasis in Zambia and characterization of *Trypanosoma brucei rhodesiense*

Chihiro Sugimoto
Dept. of Education and Collaboration
Hokkaido University Research Center for Zoonosis Control

HAT is a tsetse-transmitted neglected tropical disease threatening over 60 million people in sub-Saharan Africa (Fevre et al., 2005). HAT epidemics have been reported in Zambia's neighboring countries (WHO, 2006). Although there are several unpublished HAT cases, there is paucity of published data on the current prevalence of HAT in Zambia, mainly because HAT is thought to claim fewer lives annually compared to malaria or tuberculosis. However, HAT could be under-reported but likely to be significant among remote rural Zambians in tsetse-infested Luangwa valley. Furthermore, most of the HAT cases could be misdiagnosed for malaria or other disseases due to similarities in presenting signs. Hence there is urgent need for HAT surveillance in Zambia. The main objective of our activity in Zambia is thus to determine the prevalence, routes of transmission and major reservoir hosts of HAT in Luangwa valley, Zambia.

Trypanosomal parasites were detected in blood from patients with a fever of unknown origin had been admitted to the hospital in Lusaka in Jan.2009, and Jan. 2011. Parasites were isolated from the blood sample by inoculation into a mouse and identified as *T. brucei rhodesiense* based on the presence of serum resistance-associated antigen (SRA) gene. We conducted surveillance of HAT in 2009 and 2010 in the Eastern Province of Zambia where the patient had been living. Several SRA-positive parasites were detected in samples of bovine blood and tsetse flies (*Glossina palpalis*). Furthermore, 3 strains were isolated from mice inoculated with salivary glands of tsetse flies, which were revealed to be SRA-positive. Genetic similarities among the isolate from the patient and those from tsetse flies were demonstrate by the microsatellite marker analysis.

The result of this study raises further questions about the epidemiology of human African trypanosomiasis in Zambia.

1) Are the current epidemic strains genetically close to those isolated in 1980s'?
2) Are they genetically related to the isolates from other Eastern/Southern African countries?
3) How wild and domestic animals play role in epidemiology of HAT?
4) Are there any other infection foci in Zambia?
5) Are there SRA-negative parasites which are infective to humans?

Collaborations:
Dr. Noboru Inoue, National Reserarch Center for Protozoan Diseases, Obihiro University
Dr. Boniface Namangala, School of Veterinary Medicine, University of Zambia

Characterization of hantaviruses in Mexico and construction of infectious cDNA clone of tick-borne encephalitis virus

Hiroaki Kariwa
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Graduate School of Veterinary Medicine, Hokkaido University

The following studies were performed in hantavirus infection and tick-borne encephalitis.

Characterization of hantaviruses in Mexican wild rodents

Hantavirus causes two forms of severe human diseases, which are hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS). To know the genetic information of hantaviruses in Mexico, viral S, M and L genes in rodent lungs were amplified and nucleotide sequences of the open reading frames were determined. The results indicate that there are at least 3 distinct hantaviruses in Mexico, which were designated as Montano virus (MTNV) from *Peromyscus beatae*, Carrizal virus (CARV) from *Reithrodontomys sumichrasti* and Huitzilac virus (HUIV) from *R. megalotis*. Antigenic characterization of Mexican hantaviruses was also attempted by using immune sera to recombinant hantavirus nucleocapsid protein (NP) and monoclonal antibodies to MTNV NP. It is suggested that the antigenic properties of Mexican hantavirus NPs are quite similar each other and cross reacted with NP of Sin Nombre virus which is a causative agent of HPS in North America.

Construction of an infectious cDNA clone of the Sofjin strain of tick-borne encephalitis virus

Tick-borne encephalitis virus (TBEV) causes severe viral encephalitis in humans. In our previous studies, the Far-Eastern subtype of TBEV was isolated in Hokkaido. To identify the viral molecular determinants involved in TBEV pathogenesis, we constructed an infectious cDNA clone of the Sofjin strain, the prototype strain of the Far-Eastern subtype. The recombinant viruses derived from the infectious cDNA were shown to cause a severe encephalitic disease in a mouse model. The infectious cDNA can be used to reveal the pathogenesis of TBEV.

Study on genetic factors conferring resistance and susceptibility to zoonosis in mice

Takashi Agui
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Department of Disease Control
Graduate School of Veterinary Medicine, Hokkaido University

1. Study on the mouse Oas1b

The Oas1b is an anti-flavivirus factor induced by type I interferon. It has been reported that most of the laboratory mice such as BALAB/c, C57BL/6 (B6) etc. are deficient in Oas1b function due to the nonsense mutation of the *Oas1b* gene. The anti-flavivirus activity of the Oas families is thought to be achieved by oligo adenylates produced by Oas enzymatic activity, followed by the activation of RNase L and degradation of viral RNA. However, recent several limes of evidence showed that mouse Oas1b does not possess enzymatic activity to produce oligo adenylates. Thus, we attempted to elucidate the possible mechanism of anti-flavivirus activity of mouse Oas1b.

We developed *in vitro* assay system that the anti-flavivirus activity can be easily assessed using a West Nile virus (WNV) replicon attached with a reporter gene, secreted alkaline phosphatase (SEAP). Using this system, B6 type of Oas1b, in which nonsense mutation is present, is surprisingly shown to inhibit replication of the WNV replicon as well as wild type of Oas1b derived from feral mouse-derived inbred strain, MSM. This result suggests that B6 type of Oas1b itself possesses antiviral activity, although expression of the Oas1b is repressed upon infection by the nonsense-mediated decay *in vivo*, which may be the reason why B6 mice are susceptible to WNV infection.

2. Quantitative trait locus (QTL) analysis in resistance and susceptibility to echinococcosis in mice

It has been reported that B6 and DBA/2 (D2) mice are resistant and susceptible to primary alveolar echinococcosis, respectively. Thus, we performed QTL analyses to attempt the identification of genetic factors to determine resistance/susceptibility to echinococcosis. We performed QTL analysis using backcrosses, (B6 x D2)F_1 x D2, with respect to the two types of QTs, the number of parasite legions at 2 weeks post infection of 200 eggs (QTL 1) and the number of mature protoscolex in liver at 16 weeks post infection of the same number of eggs (QTL 2). QTL analyses gave us successful results that a significant and a highly significant QTLs were detected at the vicinity of microsatellite markers, *D6Mit150* and *D1Mit14* in QTL 1 and 2, respectively. These results lead to the identification of genes responsible for resistance/susceptibility to echinococcosis and the development of therapeutics of echinococcosis in human.

Studies on epidemiology, pathogenesis and drug development of zoonotic parasitic diseases

Ken Katakura
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Graduate School of Veterinary Medicine, Hokkaido University

1. Epidemiological studies of parasitic diseases in livestock in Myanmar

Parasitic diseases of livestock were investigated in three regions in Myanmar in 2009 and 2010.

A total of 521 cattle blood DNA samples were examined by PCR or nested PCR for piroplasm infections. Prevalences of *Babesia bovis*, *B. bigemina*, and *Theileria orientalis* were 20.7, 36.7, and 49.5%, respectively. *Rhipicehalus* (*Boophilus*) *microplus* and *Haemaphysalis* spp. were collected from the cattle. Prevalences of *B. bovis* and *B. bigemina* by nested PCR was 20.1 and 30.2%, respectively, in a total 90 *Boophilus* ticks, suggesting a vectorial role of this tick in the transmission of *Babesia* parasites. However, *Theileria* parasites were not detected in the *Haemaphysalis* ticks by PCR.

Sero-prevalences of *Neospora* and *Toxoplasma* infections was 10.7% by ELIZA and 1.6% by Latex-aggutination test, respectively, in a total of 569 cattle.

Loop-mediated isothermal amplification was used to detect *Trypanosoma evansi* infection in a total of 87 blood DNA samples collected from 30 horses, 18 donkeys, and 39 mules. One donkey sample was positive, suggesting the presence of *T. evansi* in equids in Myanmar. As potential vectors, three *Tabanus*, one *Chrysops*, three *Stomoxys*, one *Haematobia*, and one *Haematobosca* species were identiflied.

2. Involvement of $CD4^+Foxp3^+$ regulatory T cells in the persistent infection of *Leishmania donovani* in mice

Alymphoplasia (*aly/aly*) mice with a mutation of NF-kB inducing kinase gene were used as a chronic visceral leishmaniasis model. Although *Leishmania* parasites were almost eliminated in control (*aly/+*) mice by 4 months of post infection, the parasites persisted in the liver, spleen and bone marrow of *aly/aly* mice. Increases of the number of $CD4^+Foxp3^+$ T cells and Foxp3 mRNA level were obseved in the liver of *aly/aly* mice. Intraperitoneal injection of the infected-mice with anti-CD25 or anti-FR4 antibodies at 6.5 months of post infection revealed a significantly reduction of parasite burdens in these organs, suggesting that $CD4^+Foxp3^+$ Treg cells may play a significant role in the persistence of *L. donovani* parasites in this murine model.

Laboratory and Epidemiological Investigations of Leptospirosis and Rabies in Kandy, Sri Lanka, 2009-2010

Hiko Tamashiro
Department of Global Health and Epidemiology
Hokkaido University Graduate School of Medicine

Introduction:

I will present our research activities on leptospirosis and rabies in Kandy, Sri Lanka for 2009-2010.

Materials and Methods:

- The nation-wide sentinel surveillance data on leptospirosis were collected from the Ministry of Health from 2005 to 2008 to depict the dynamics of leptospira infection.
- Circulating leptospiral serogroups and species in febrile patients and community people were analyzed in the blood samples and epidemiological data were collected from patients at the University of Peradeniya Teaching Hospital (UPTH) and community residents.
- Prevalence and carrier status in smallholder dairy cattle and rodents were studied and other specimens were analyzed using MAT and PCR.

Results:

- *Dynamics of leptospira infection:* Of 4000 suspected cases, most were men (83.5%) and aged 30 -49 years (45.6%). 16.5% were farmers and 16.1% were laborers and nearly half (44.8%) had no information on occupation.
- *Circulating leptosprial serogroups and species:* Of 97 cases, 25 were confirmed as leptospiral cases by MAT or PCR. The predominant serogroup was sejroe. *L.* DNA was identified as *L.* interrogans.
- *Prevalence and carrier status in smallholder dairy cattle and rodents:* The cattle in 19 (38.8%) of the 49 farms harbored anti-leptospiral antibodies. Out of 113 specimens, 23 were positive; 17 and 6 reacted with serogroups sejroe and hebdomadis, respectively. Out of the 74 rodent specimens, 13 were positive; 8 and 4 had reactions to serogroups Javanica and Icterohaemorrhagiae, respectively. *L.* DNA was identified as *L.* interrogans.
- The Rabies Control Unit (RCU) at UP continues to diagnose suspected rabies animals in Kandy and the neighboring Districts. In 2010, 81 specimens were submitted to RCU by government sectors and the general public, a decrease of 19.0% from 2009. Among the specimens, 21 (25.9%) were positive on rabies and canine rabies accounted for 30% of the submitted dog specimens (18 out of 60). In addition, with RCU, local health government and community representatives, a pilot serological survey among sheltered dogs was carried out to detect the rabies virus antibody levels using the anti-rabies vaccination. The survey revealed that puppies under three months of age might need a vaccination with booster.

Conclusions:

Our research activities have revealed scarcely-known evidence on the dynamics and epidemiology related to leptospirosis and rabies in Kandy, Sri Lanka. More studies are needed to further examine these zoonotic diseases towards their efficacious control and prevention.

Clinical Study of Human Bocavirus Infection

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Human bocavirus (HBoV), belonging to the family *Parvoviridae*, subfamily *Parvovirinae*, and genus *Bocavirus*, was cloned by molecular screening of pooled human respiratory tract samples in 2005. We have clarified the following. (1) HBoV was detected by PCR from nasopharyngeal swab samples (8 of 318; 5.7%) collected from children with lower respiratory tract infections (RTIs). (2) The overall seroprevalence rate of antibodies against the VP1 protein of HBoV in a Japanese population aged from 0 months to 41 years was 71.1% (145 of 204). (3) The seropositive rate was lowest in the age group of 6 to 8 months and gradually increased with age. All of the children had been exposed to HBoV by the age of 6 years.

A 14-month-old boy, previously healthy, was admitted to hospital (Dept. of Pediatrics, Chiba university hospital) with cough, fever, and increasing dyspnea, and was diagnosed as plastic bronchitis. The intratracheal aspirate was subjected to real-time PCR screening for bacterial and viral pathogens, and only HBoV was detected. HBoV DNA was detected in the acute phase (2.10×10^4 copies/mL) but not in the convalescent phase ($<4.0 \times 10^2$ copies/mL). The titers of the IgG antibody against HBoV VP1 increased from <1:40 (acute phase) to 1:2560 (convalescent phase). Our data support the hypothesis that HBoV causes RTI in children.

Recently, novel bocaviruses (HBoV2, HBoV3 and HBoV4) were discovered in fecal samples collected from gastroenteritis patients. We are investigating the prevalences of these novel bocaviruses in nasopharyngeal samples. Results will be reported in the GCOE seminar.

Toward the Control of Viral Zoonoses
“Influenza virus and Filovirus”

Ayato Takada
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Recently, emerging and reemerging infectious diseases, such as SARS, Nipah, Hanta, Hendra, Influenza, Arena and Ebola virus infections, are appearing worldwide, and becoming of major concerns to public health. All of these viral diseases are zoonoses whose causative agents infect both humans and animals. The agents are originally harmless in their natural host wild animals and occasionally transmit to other animal species including humans, causing infectious diseases. Changes in the global environment and human behavior likely contribute to the emergence of new diseases by bringing people into closer and more frequent contact with pathogens. In addition, the increase of international travelers and animal trade has also contributed to a rise in opportunities for pathogens to jump from natural host animals to humans. For the establishment of preemptive measures against such zoonoses, a prerequisite is to identify natural host animals carrying potential pathogens and to elucidate the transmission routes and factors involved in the spread and pathogenesis of infections. In this symposium, our research activities on influenza virus and filovirus epidemiology will be presented.

Research on wildlife ecology and zoonosis control

Toshio Tsubota
Laboratory of Wildlife Biology and Medicine, Graduate School of Veterinary Medicine,
Hokkaido University

Wildlife often plays a key role in emerging and re-emerging zoonosis. Some creatures act as natural hosts of zoonoses in nature by maintaining pathogens, and new routes of infection are often established as a result of ecosystem imbalance and exploitation. The goal of the present study is therefore to clarify the relationships between wildlife ecology and zoonosis infection in nature. The findings of the study in this fiscal year is as following.

1. Relationship between biodiversity and prevalence of Lyme disease in wildlife of Hokkaido

Lyme disease is a zoonotic disease caused by *Borrelia burgdorferi* sensu lato and transmitted by ticks of the genus *Ixodes*. With a wider scope of increasing baseline knowledge of the interactions among wildlife, *Ixodes* ticks and *B. burgdorferi*, the initial hypothesis of our study is that areas of greater wildlife diversity in Hokkaido have lower borrelia infection rates. We chose to focus attention on rodents as representatives of diversity and as the most likely reservoirs of *borrelia*.

Between May and September of 2010, we trapped, in a total of 7,440 trap nights, 296 rodents from two locations in Hokkaido, one in eastern (Area 1) and one in central Hokkaido (Area 2). Rodent abundance estimates showed that Area 1 had a greater number of rodents across all months. Whereas the species types trapped were similar, species ratios were significantly different for *Myodes rufocanus* and *Apodemus argenteus*. DNA was extracted from urinary bladders of all rodent samples, and polymerase chain reaction (PCR) was used to amplify part of the flagellin gene of *Borrelia* spp. Positive reactions were attained for 73 samples (Area 1: 21.3% and Area 2: 41.7%), and the amplified DNA was sequenced to reveal four species of *borrelia*: *B. garinii*, *B. afzelii, B. miyamotoi* and *B. japonica*. It was evident that *borrelia* infections, all counted together, were more likely to occur when ticks were found attached to rodents ($P<0.01$).

2. Comparison of tuberculosis prevalence between 2 areas of National Parks (NPs) in Zambia

Tuberculosis (TB) is a zoonotic disease caused by *Micobacterium* spp. and can be transmitted among wildlife, domestic animals and human beings. Kafue lechwe, a lechwe subspecies and inhabits the area around Lochinvar NP, indicated positive of *M. bovis* and is regarded as a reservoir of TB. In this fiscal year, we tried to detect *M. bovis* in cattle in Mongu where is close to Liuwa floodplain NP as a preliminary study for the investigation of TB prevalence in red lechwe, a lechwe subspecies and inhabits the area around Liuwa floodplain NP. We are examining to culture *M. bovis* colonies and to identify their DNAs by LAMP method. Relating this TB study in lechwe, we are also examining the TB of captive sika deer occurred in Osaka last January.

Studies on diagnoses, epidemiology and pathogeneses of hantavirus infection

Jiro Arikawa
Department of Microbiology, Division of Infectious Diseases,
Graduate School of Medicine, Hokkaido University

Hantaviruses are causative agents of two important rodent-borne zoonoses, hemorrhagic fever with renal syndrome (HFRS) in Eurasia and hantavirus pulmonary syndrome (HPS) in North and South America. In a GCOE program, diagnoses, epidemiology and pathogeneses of hantavirus infection have been studied as research subjects. In this symposium, recent progress in the following subjects will be presented.

1. Diagnoses: *E. coli*-expressed entire hantavirus recombinant nucleocapsid protein (rNP) and baculovirus expressed N-terminal truncated rNP were developed as ELISA antigens for screening and serotyping of hantavirus infection, respectively. Availability of the two ELISAs was confirmed by examining numbers of patient or animal sera those were infected with HFRS or HPS causing viruses. Acute phase HPS patient sera were serotyped by the serotyping ELISA, indicating the availability for early diagnoses.

2. Epidemiology: Collaboration study with institutes in Viet Nam, Indonesia, Thailand, India and Sri Lanka elucidated that two types of hantaviruses, Seoul virus (SEOV) and Thailand virus (THAIV) were circulating in both humans and rodents in East and South East Asian countries. Antibody positive rates between fever patients of unknown origin and ordinary people were about 2% in both groups, indicating that the virulency of the hantavirus in East Asian countries might be low. Shrew borne hantavirus, Thottapalayam virus (TPMV), antibody positive sera were found out in both humans and shrew in Viet Nam, Indonesia and Thailand suggests the existence of variety of hantaviruses in Asian countries. Further epidemiological and epizootiological studies are required to clarify distribution, variation and consequence as a cause of HFRS.

3. Studies on pathogenesis of Seoul virus infected rats: The mechanism by which causing persistent infection in natural reservoir rodents has been studied by comparing mode of infection between SEOV infected laboratory rats and naturally infected Norway rats captured at endemic country. For the immunological characterizations, mitogenic activity to ConA and virus specific cytotoxic T lymphocyte assays were established by using infected laboratory rats. These methods will be applied to naturally infected Norway rats to examine mechanisms for persistent infection in the natural host.

Epidemiological study of avian infectious diseases

Kazuhiko Ohashi
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Graduate School of Veterinary Medicine, Hokkaido University

Wild birds play an important role as reservoirs in the introduction/transmission of several infectious diseases including zoonosis such as West Nile virus (WNV) and Japanese encephalitis virus (JEV) infections. In this study, molecular epidemiological and serological survey methods using feather tips of birds will be developed to conduct the survey of these infectious diseases in the Hokkaido area. Molecular epidemiological survey method based on RT-PCR established showed that, thus far, no positive birds were found in the survey of 2010 in the Hokkaido area. An attempt was made to develop serological method to detect specific antibodies to pathogens using feather tips. Western blot analyses were performed to detect IgG proteins extracted from feather tips of wild birds using several commercial antibodies to avian IgG. Heavy and light chains of IgG were detected in most bird species using rabbit anti-chicken IgG antibodies, showing that feather tips can be used for the serological survey of wild birds. Thus, a serological method to detect WNV- or JEV-specific antibody from feather tips of wild birds will be constructed as a next step.

Marek's disease virus (MDV), which causes a neoplastic disease in domestic chickens, is also widely present in wild waterfowls, and the increase in its virulence has been reported for the last decade. When the diversities in a candidate oncogene of MDV (i.e. *meq*) were monitored in the recent field isolates of MDV of 2009-2010, mutations potentially related to the drastic increase in its virulence have not been found, though several mutations have been observed in these isolates.

The poultry red mite, *Dermanyssus gallinae*, distributed worldwide, is an economically important parasitic pest of domestic chickens. The red mite has been also suggested as a potential vector of several pathogens. However, little is known on the molecules of the mite, and acaricide-resistant mite is also widely present in farms, which could be a serious problem to poultry industry in the future. Therefore, a global analysis of gene expressions in the red mite has been performed. A total of about 1,000 expression sequence tags (ESTs) were sequenced from the red mite cDNA library. Many of these clones have high-homology with the genes of *Ixodes scapularis*, known as a vector of the Lyme disease. In addition, one clone was found to be homologous to antioxidant, thioredoxin peroxidase, which is suggested as a possible vaccine candidate for other ticks. Currently, functional analysis of these clones is in progress to identify new vaccine candidates and target molecules for acaricide.

Prediction of amino acid substitutions on the hemagglutinin molecules of antigenic variants of influenza A viruses

Kimihito Ito
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Hokkaido University Research Center for Zoonosis Control

Human influenza A viruses undergo antigenic changes with gradual accumulation of amino acid substitutions on the hemagglutinin (HA) molecule. A strong antigenic mismatch between vaccine and epidemic strains often requires the replacement of influenza vaccines worldwide.

To establish a practical model enabling us to predict the future direction of the influenza virus evolution, relative distances of amino acid sequences among past epidemic strains were analyzed by multidimensional scaling (MDS). We found that human influenza viruses have evolved along a gnarled evolutionary pathway with an approximately constant curvature in the MDS-constructed 3D space. The gnarled pathway indicated that evolution on the trunk favored multiple substitutions at the same amino acid positions on HA.

We also found that a mathematical model could predict the relative sequence distances among past epidemic strains. Retrospective tests for 12 years showed that the model could predict the direction of the evolution of human H3N2 viruses with high accuracy. Retrospective tests also highlighted an unresolved issue that our method could not predict the exact timing when a dominant strain was replaced by another strain. However, the tests showed that the method could have picked proper vaccine strains earlier than the announcement of the reformulation of vaccine strains by WHO, indicating its potential to recognize the new strains before they become dominant.

In this presentation, I am going to discuss the past, current and future evolution of influenza A viruses through these bioinformatics technologies.

Towards understanding of pathogenesis of prion infection

Motohiro Horiuchi
Laboratory of Veterinary Hygiene, Graduate School of Veterinary Medicine,
Hokkaido University

Prion diseases are characterized by the accumulation of abnormal isoform of prion protein (PrP^{Sc}) in central nervous system, neuronal loss, astrogliosis and microglial activation. Prion propagation evokes microgilal and astrocyte activation and finally causes neuronal degeneration, however, how microglia and astrocytes recognize and respond to prion propagation and the mechanism of neuronal death by prion propagation remain to be elucidated. To analyze the pathogenesis of prion infection, this year, we attempted to establish 1) multiple fluorescence staining for the detection of PrP^{Sc} and marker antigens in frozen brain section, and 2) prion-infected primary neural culture obtained from differentiated mouse neurospheres.

Difficulty in the distinction of PrP^{Sc} from cellular isoform of prion protein (PrP^{C}) was one of the major technical obstacles for analyses of pathogenesis of prion infection. So far, no antibodies that can distinguish PrP^{Sc} from PrP^{C} in immunohistochemistry under non-denatured condition are available. Recently we showed that mAb 132, recognizing the most amyloidgenic region of PrP molecule, is useful for specific detection of PrP^{Sc} in prion-infected neuroblastoma cells. Although it requires pretreatment of cells with chaotropic reagent, mAb132 enables us to detect PrP^{Sc} reproducible and reliable. Therefore, we attempted to adopt mAb132 for the detection of PrP^{Sc} in frozen tissue section. After the repeated refinement of protocol, we optimized the double-staining of PrP^{Sc} with neural marker proteins, and are now analyzing the brain regions where PrP^{Sc} is detected in the early stage of infection and the microglial and astrocyte responses in those regions.

Neuroblastoma cells persistently infected with prions are widely used for the analysis of prion propagation. However, no apparent neuronal death or damage is observed in the neuroblastoma cells. Although prion-infected neuroblastoma cells are indispensable tool for the analysis of prion propagation, neuroblastoma cells may not be a suitable model for neuronal degeneration caused by prion propagation. To establish ex vivo culture system in which neuronal death or degeneration by prion propagation can be reproduced, we attempted to use mouse neurospheres and characterized prion infection in differentiated neurospheres. PrP^{Sc} was preferentially observed GFAP-positive astrocytes, however, PrP^{Sc}-positive neurons were observed after 18 days post infection and then gradually increased. Prion-infected primary neural cultures from differentiated neurospheres could be maintained over 50 days, and several pre-and post-synaptic markers were expressed with differentiation. Thus this ex vivo system would be useful for the analysis of neuronal death and neuronal degeneration caused by prion infection.

Investigation of viral release mechanism of Polyomavirus and Molecular Epidemiological Research of Infectious Diseases in Zambia

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We have recently reported that novel viral release mechanism of Polyomavirus from infected cells.

Viroporins are a group of proteins that participate in the promotion of release of viral particles from cells, and interact with cellular membranes modifying permeability. These proteins are not essential for the replication of viruses, but their presence often enhances virus growth. Here, we demonstrate that the JC virus agnoprotein forms homo-oligomers as an integral membrane protein and acts as a viroporin, and that expression of agnoprotein results in plasma membrane permeabilization and virion release. These observations suggest that the virion release process of a non-enveloped DNA virus is highly regulated by a single viral protein. This work was mainly performed by Dr. Tadaki Suzuki who was the post doc in the Research Center for Zoonosis Control and now worked in the National Institute of Infectious Diseases, Murayama, Tokyo.

We also performed the molecular epidemiological research of infectious diseases in Zambia.

We have examined retroviruses in non-human primates in Zambia and isolated simian immunodeficiency virus from peripheral blood mononuclear cells. This work was mainly performed by Dr. Akira Kawaguchi who was the Ph. D. student and now worked in the National Institute of Infectious Diseases, Murayama, Tokyo and Dr. Akihiro Ishii who is the assistant professor in the Research Center for Zoonosis Control.

As for rabies, we performed phylogenetic analysis of rabies in Zambia. The rabies virus in Zambia belongs to the Africa 1b lineage. This work was mainly performed by Dr. Walter Muleya who is the 2nd year Ph. D. course student.

The origin of antibodies in the cerebrospinal fluids by intrathecal immunization

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Intrathecal (IT) immunization is a vaccination by injecting antigens directory into subarachnoid spaces including ventricles and brain parenchyma. We previously reported that the vaccination induced antigen-specific antibodies not only in the serum but also in the cerebrospinal fluid (CSF), and intrathecally immunized mice were completely protected against pseudorabies and rabies virus challenges in peripheral tissue. Furthermore, 80% of intrathecally immunized mice tolerated intracerebral rabies virus challenge without any clinical signs.

To better understand the immune mechanisms of IT immunization, immune responses in both the central nervous system (CNS) and lymphoid organs following the immunization using inactivated pseudorabies virus were investigated by focusing on antibody secreting cells (ASCs) and the role of chemokines for attraction of ASCs to the CNS to suppress replication of a neurotropic virus. The spleen and cervical lymph nodes of IT immunized mice produced significantly more virus-specific antibodies than those of subcutaneously (SC) immunized mice. ASCs, immunoglobulins and mRNAs of IgG, CXCL9, 10, 13 and BUFF were predominantly detected in the brains of intrathecally immunized mice, but not in SC immunized mice, and the migration of the cells in the CNS was stimulated by chemokines, predominantly CXCL12 and cocktail chemokines. Furthermore, the injection of these chemokines extended the survival time of ASCs in the CNS. These results suggested that attraction of ASCs into the CNS following the upregulation of chemokines contributes to eliminating the neurotropic virus.

Antibody titers in the CSF by repeated IT immunization did not exceed those of serum, and SC immunization prior to IT immunization resulted in induction of higher and more prompt antibody response in the CSF than IT immunization alone. These results suggested that the CSF antibody induced by IT immunization originated both from antibodies of peripheral blood and de novo antibodies locally produced in the CNS. These informations indicate us new and promising strategies of IT immunization for treatment of rabid animals and humans.

Analysis of immune responses in infectious diseases

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NKT cell as a target for controlling infectious diseases

Natural killer T (NKT) cells has been recognized as a unique subset of T-lineage cells that recognize various lipid antigens (Ag) in the context of CD1d molecules. Amongst lipid Ag listed to date, α-galactosylceramide (α-GC) is the most prominent ligand for invariant NKT cells and can induce vast arrays of Th1-, Th2- or Th17-type cytokines upon stimulation. CD1d-restricted, invariant NKT cells seem to be devised as one of the warriors against microbial infections *in vivo*, since $CD1d^{-/-}$ mice are sensitive to certain pathogenic microbes and the stimulation with ligands demonstrates beneficial effects against the infections in wild type (WT) mice. Actually, invariant TCR can recognize α-galacturonylceramide from $Gram^{-}$ lipopolysaccharide $(LPS)^{-}$ bacteria, *Sphingomonas spp*, and also α-galactosyldiacylglycerol from a spirochete, *Borrelia burgdorferi* that causes Lyme disease, both in CD1d-restricted fashion. NKT cells can also sense LPS in antigen presenting cell (APC)-dependent manner through either recognition of induced (self) lipid Ag in the context of CD1d or signals of soluble factors (IL-12 and IL-18) even in the absence of CD1d. Through dual modes of recognition, NKT cells can monitor infection by a wide variety of pathogens and act as innate effector cells not only to initiate early responses but also to enhance following acquired immune responses with abundant productions of various interleukins. This advantage with NKT cell should be actively taken for immunopotentiation, since specific (more Th1- or -2-biased) ligands have been developed. Of note, NKT cells also recognize endogenous or food-derived lipid ligands *in vivo* probably as pathogen-derived ones in a cross-reactive way, and are activated in hyperlipidemic state induced by feeding high fat diet. Thus, the host environment such as obesity may influence immune responses.

Mucosa-associated invariant T (MAIT) cell also constitutes an innate T-cell that possesses invariant TCR Vα chain (Vα19Jα33 in mouse and Vα7.2Jα33 in human) and expresses NK1.1. Thus, MAIT cells can be recognized as a subset of NKT cells. MAIT cell, as originally identified as an important subset in mucosal immunity by its name, has also been demonstrated as regulatory cell for an inflammation in central nervous system with the potent IL-10-producing activity. Thus, the MR1-restricted NKT cell may have more regulatory nature than the counterpart, CD1d-restricted NKT cell. Recently however, it has been shown that MAIT cells were stimulated with APC infected with *M. tuberculosis*, *E. coli* and other bacteria, suggesting that MAIT cells recognize a common signature presented by MR1 molecules. Another subset of NKT cells is also involved in host defense against infectious agents, although the nature of Ag, the mode of presentation, and the non-overlapping functions with CD1d-restricted NKT cells remain elusive.

As a model to analyze immune/inflammatory response, we examined the development of atherosclerosis in either $CD1d^{-/-}$ or $MR1^{-/-}$ mice, and found the differential pathology in respective strains of mice. The lesion development of atherosclerosis was ameliorated in $CD1d^{-/-}$ mice and aggravated in $MR1^{-/-}$ mice when compared respectively to WT. Moreover in $MR1^{-/-}$ mice, CD1d-restricted NKT cells were activated *in vivo* with time, suggesting that MAIT cells possess a regulatory role on the activity of CD1d-restricted NKT cells.

The Intramolecular Element Responsible for Reduced Expression of Borna Disease Virus Glycoprotein in Transfected Cells

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Borna disease virus (BDV) causes Borna disease, a frequently fatal meningoencephalitis with behavioral abnormalities and diverse pathology in several vertebrates. While the virus envelope glycoprotein (BDVG) has been believed to play pivotal roles in BDV infection and pathogenesis of the disease in the central nervous system, the mechanisms for cell entry, cell tropism, and propagation of BDV remain unknown. In the present study, we analyzed expression and localization of BDVG, ABVG, avian bornavirus glycoprotein, and their chimeric proteins in transfected HEK293 and Vero cells, using immunofluorescent microscopy, immunoblotting, and cell fusion assay.

In remarkable contrast to that the control VSVG showed abundant and predominant localization at the plasma membrane, both BDVG and ABVG principally had intracellular distribution, suggesting the ER retardation of these glycoproteins. Particularly, BDVG exhibited an extremely reduced level of expression, while ABVG was readily detected in immunoblotting and immunofluorescence. Analysis of a series of chimeras of BDVG and ABVG demonstrated that differential expression of these viral glycoproteins was derived from the amino acid sequence in a part of the transmembrane span that differed each other between BDVG and ABVG. On the other hand, fusion assay of Vero cells transfected with these glycoproteins revealed that low level expression of these envelope proteins were sufficient to cause the cell-to-cell interaction.

These findings suggest that reduced expression of viral glycoproteins of BDV is relevant to persistent infection of the virus and progressive pathology of the disease. Possible mechanisms may involve interaction of BDVG with the ER-resident chaperone calnexin through peptide-based association as we found for the interaction between a mutant of AE1 and calnexin. Recognition of more than ten *N*-glycan chains attached to BDVG by calnexin may also participate in ER retardation and reduced expression of BDVG.

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Easy and rapid detection of *Mycobacterium tuberculosis* by a newly developed isothermal nucleic-acid amplification method targeting tandem repeat sequences

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Mycobacterium tuberculosis is the causative agent for tuberculosis (TB) and it has been the most important bacterial pathogen causing about two million deaths around the world every year. Since the growth rate of *M. tuberculosis* is significantly slow, the development of rapid diagnostic methods using molecular techniques is highly needed. Considering the situation that higher infection and mortality rates are especially observed in developing countries, TB diagnosis methods should be simple and easy to perform without the requirement of expensive instruments.

A pair of primers was designed to recognize the direct repeat (DR) sequence on *M. tuberculosis* complex (MTC) genomes. Reaction mixture was prepared according to the loop-mediated isothermal amplification (LAMP) method with *Bst* DNA polymerase and the reaction was performed under isothermal condition. An appropriate condition was determined with MTC and non-tuberculous mycobacteria (NTM) samples. By an isothermal incubation at 68 °C, *M. bovis* BCG strain or *M. tuberculosis* were successfully detected. The detection limit of DNA of both *M. bovis* BCG strain and *M. tuberculosis* clinical isolates were 50 fg/tube, equivalent to 10 bacterial cells, within 60 min. Under this reagent and temperature condition, no NTM samples turned positive during 120 min incubation. Those reactions could be detected by naked eye with the addition of fluorescent dye.

In current study, we developed a new nucleic acid amplification method that can be conducted under an isothermal condition. A water bath or a heat block is only required equipment for this method. The sensitivity and specificity were high and comparable to those of LAMP method. Additionally, only two primers are necessary in this method whereas LAMP requires 6 primers, suggesting an advantage in cost. This easy, rapid and low-cost method can be a first choice for TB detection in resource-limited laboratories in developing countries.

Characterization of the roles of the interaction of influenza virus NS1 with Akt

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The PI3K-Akt network, which is activated by cytokines or growth factors, mediates intracellular signals to regulate a variety of cellular responses, including antiapoptosis, proliferation, cell cycling, protein synthesis, glucose metabolism, and telomere activity. Genomic mutations, alterations of the PI3K-Akt regulatory network, underlie such diseases as cancer, glucose intolerance (diabetes mellitus), schizophrenia, and/or autoimmune diseases. In addition to direct tumorigenesis involvement by genetically altering human cancer, the PI3K-Akt network underlies the clinical manifestation of different stages of tumorigenic viral infection, such as latent and chronic infection, and malignant transformation of Epstein-Barr, hepatitis C, hepatitis B, and human immunodeficiency virus (HIV) viruses. Host defense against viruses depends on the targeted cell death of infected host cells and subsequent cellular clearance by macrophages. By effectively activating the PI3K-AKT network, virally infected cells can presumably avoid their own virus-induced death.

Here in we investigated the functional interaction of NS1 with serine threonine kinase Akt, a core intra-cellular survival regulator. In co-immunoprecipitation assays and GST pull-down assays, NS1 directly interacted with Akt. The interaction was mediated primarily through the Akt-PH (Pleckstrin Homology) domain and the RNA- binding domain of NS1. NS1 preferentially interacted with phosphorylated Akt, but not with non-phosphorylated Akt. Functionally, the NS1-Akt interaction enhanced Akt kinase activity both in the intra-cellular context and in in vitro Akt kinase assays. Confocal microscopic analysis revealed that phosphorylated Akt interacted with NS1 during the interphase of the cell cycle predominantly within the nucleus. Finally, mass spectrometric analysis demonstrated the position at Thr215 of NS1 protein is primary phosphorylation target site through Akt activation. The results together supported the functional importance of influenza virus NS1 with Akt, a core intra-cellular survival regulator.

Antiapoptotic PI3K-AKT signaling may, thus, allow scavengers to control the host cell-to-cell infectious process. Observation will help to facilitate the development of pharmacological compounds for AKT-NS1as a possible molecular target for influenza infection.

Mechanism for the pathogenesis of influenza and development of antiviral drug

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Type I interferons (IFNs) are critical for the host defense against influenza A virus (IAV) infection. Recently, it is shown that IFN induction mediated by RIG-I and IPS-1 is important for antiviral responses for IAV. Nonstructural protein 1 (NS1) of IAV was previously demonstrated to inhibit this IFN induction pathway. We found that RNA polymerase complex of IAV inhibited the IFN induction mediated by RIG-I and IPS-1, and this activity was not competitive with the function of NS1. Each polymerase subunit, PA, PB1 or PB2 was associated with IPS-1, and each subunit inhibited the activation of IFNβ promoter by IPS-1 independently. Activation of IFNβ promoter mediated by IPS-1 was more efficiently inhibited by PB2 or the complex containing PB2. These results demonstrate that the viral polymerase plays an important role for the inhibition of IFN production by the association with IPS-1.

After influenza A virus infection, a variety of inflammatory cells are recruited into the virus-infected sites as a critical step of host defense response. This process is tightly coordinated by the interaction of cells with their surrounding extracellular matrix (ECM) proteins.

In addition, matrix metalloproteinases (MMPs) and their inhibitors play an important regulatory role in the inflammatory responses. However, the functional role of these molecules (ECMs, cell adhesion molecules, MMPs and MMP inhibitors) after the viral infection is still unclear. We found alterations of gene expression of several MMPs and ECMs in the lungs of mice infected with influenza A virus. In particular, the mRNA expression of tissue inhibitor of metalloproteinase-1 (TIMP-1), which is a specific inhibitor of MMPs, was remarkably induced in the lungs after the viral infection. Therefore, we focused on the function of TIMP-1 for the pathogenesis, and infected TIMP-1 knockout (KO) and wild type (WT) mice with the virus. After the viral infection, the survival rate of TIMP-1 KO mice was lower than that of WT mice. Therefore, it is suggested that the increased expression of TIMP-1 after influenza A virus infection is correlated to the pathological condition.

Finally, we found some Chinese herbal medicines to inhibit the plaque formation by influenza A viruses. Therefore, we purified the substances from these herbal medicines using Solvent Extraction Methods and HPLC system for the development of antiviral drug.

Structural Basis for Oncogenesis by *Helicobacter pylori* CagA

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Helicobacter pylori cagA-positive strains play a key role in the development of gastric diseases such as atrophic gastritis and gastric cancer. CagA protein is injected via the bacterial injection system into gastric epithelial cells. CagA interacts with SHP-2 and PAR1 through Glu-Pro-Ile-Tyr-Ala (EPIYA)-repeat region containing multiple EPIYA sites, which are the sites of tyrosine phosphorylation. Accordingly, CagA deregulates the cellular target molecules and perturbs multiple intracellular machineries involved in the regulation of cell morphology and cell polarity. The *cagA*-transgenic mice showed development of tumors in the gastrointestinal tract whereas the phosphorylation-resistant *cagA*-transgenic mice did not. Hence, tyrosine phosphorylation on CagA contributes to the development of gastric carcinoma caused by *cagA*-positive *H. pylori* infection and the molecular structure of EPIYA-repeat region is a participant in the oncogenic activity of CagA. To elucidate molecular basis for the CagA biological activity, we investigated tertiary structure of CagA. As results, CagA C-terminal fragment that contains EPIYA-repeat region largely consists of the unfolded structure although the fragment possesses an ability to bind with target molecule and cell morphogenetic activity that is caused by dysfunction of SHP-2. The results suggest that CagA utilizes intrinsically unfolded structure in the EPIYA-repeat region to interact with multiple cellular targets and consequently deregulates various intracellular signaling. Furthermore, we found that the C-terminal 35 kDa domain (CTD) of CagA that contains the EPIYA-repeat region shows a reduced ability to induce the morphogenetic change and that the N-terminal 100 kDa domain (NTD) physically interacts with CagA CTD. We then determined the CagA regions required for the intra-molecular interaction using a series of CagA fragments and investigated the role of the NTD-CTD interaction in the morphogenetic activity of CagA. As a consequence, we found that the CagA mutants that cannot undergo intramolecular interaction show a reduced ability to induce the morphogenetic change compared to wild-type CagA. These findings indicate that the intra-molecular association with CagA NTD potentiates the ability of CagA CTD to exert its pathophysiological actions.

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