北海道大学グローバルCOEプログラム

Global COE Program
Progress Report FY 2011
Presentation

2011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：　2012年3月8日(木)10:00～16:00

Venue: Conference Hall
Graduate School of Veterinary Medicine, Hokkaido University
Time & Date: 10:00～16:00, March 8 (thu), 2012

Global COE Program Progress Report FY 2011 Presentation

Venue: Conference Hall, Graduate School of Veterinary Medicine, Hokkaido University
Time & Date: 10:00～16：00, March 8 (thu), 2012

========== Time schedule & Contents ==========

10:00-10:05
Opening speech: Hiroshi Kida, Leader of Global COE Program

■Epidemiological Research Group■ Chairperson: Ayato Takada

10:05-11:20

1. **Recent progress on bioinformatics for the control of zoonoses**
   Kimihito Ito

2. **Toward the control of viral zoonoses "Influenza virus and filovirus"**
   Ayato Takada

3. **Research on wildlife ecology and zoonosis control**
   Toshio Tsubota

4. **Studies on diagnoses and pathogeneses of hantavirus infection and leptospirosis**
   Jiro Arikawa

5. **Epidemiological study of avian infectious diseases**
   Kazuhiko Ohashi

■Cultivation of Human Resource Group■ Chairperson: Chihiro Sugimoto

11:20-12:05

6. **Studies on clinical aspects of human bocavirus, KI polyomavirus and WU polyomavirus infection**
   Tadashi Ariga

7. **Metagenomic approach to identify tick-borne pathogens by using ultra high throughput DNA sequencing and data analyzing technologies**
   Chihiro Sugimoto

8. **Epidemiological study of hantavirus infection in Japan and characterization of tick-borne encephalitis virus isolated in Hokkaido**
   Hiroaki Kariwa

12:05-13:00 Lunch

■Cultivation of Human Resource Group■ Chairperson: Chihiro Sugimoto

13:00-13:45

9. Study on the mechanism of anti-flavivirus infection of oligoadenylate synthetase (OAS)
Takashi Agui

10. Studies on epidemiology, pathogenesis and drug development of zoonotic parasitic diseases
Ken Katakura

11. Epidemiological investigations of leptospirosis and rabies in Sri Lanka in 2011
Hiko Tamashiro

■Immunological and Pathological Research Group■ Chairperson: Motohiro Horiuchi

13:45-14:45

12. Towards understanding of pathogenesis of prion infection
Motohiro Horiuchi

13. Investigation of viral assembly mechanism of Polyomavirus and molecular epidemiological research of rabies in Zambia
Hirofumi Sawa

14. Successful treatment of rabid rabbits by intrathecal immunization
Takashi Umemura

15. Host cell factors involved in synthesis and expression of borna disease virus and avian bornavirus glycoproteins
Mutsumi Inaba

14:45-15:00 Coffee Break

■Diagnostic and Therapeutic Research Group■ Chairperson: Yasuhiko Suzuki

15:00-16:00

16. Molecular characterization of drug resistant Mycobacterium tuberculosis from Asian countries
Yasuhiko Suzuki

17. Regulation of the Akt kinase by TCL1 family oncogene TCL1b; implications for developing pharmacological reagents for Akt
Masayuki Noguchi

18. Functional role of the extracellular matrix to determine the severity of influenza
Tadaaki Miyazaki

19. Molecular-based study for the control of *Bacillus anthracis* infection
Hideaki Higashi

16:00-16:05

Closing Speech: Hiroshi Kida, Leader of Global COE Program

Recent progress on bioinformatics for the control of zoonoses

Kimihito Ito
Division of Bioinformatics,
Hokkaido University Research Center for Zoonosis Control

Through efforts combining informatics and biology, we are developing computational methods to support the prediction and prevention of the zoonotic outbreaks and epidemics. Current research topics include the prediction of antigenic changes of influenza A viruses, the analysis of sequence data obtained by next-generation sequencers.

1. Prediction of amino acid substitutions on hemagglutinin of influenza A viruses

Human influenza viruses mutate from time to time, causing seasonal influenza worldwide. To predict the amino acid sequence of an effective vaccine strain prior to each influenza season, we are investigating the evolution of HA in the past, aiming to predict the virus's evolution in the future.

Using multidimensional scaling (MDS), we analyzed relative distances of amino acid sequences among H3N2 epidemic strains isolated from 1968 to 2011. We found that two distinct H3N2 strains were co-circulating in human population in 2011. We confirmed that one strain was related to A/ Nanjing/1663/2010(H3N2), which we have predicted in December 2010. In the winter of 2011-2012 Japan has a large seasonal influenza epidemic. It has been known that the epidemic was caused by H3N2 strains, but the HA sequence of the 2012 strain has not published so far. The HA sequence the Japanese epidemic strain would help us to evaluate the accuracy of our prediction method. This result also highlighted an issue to predict exact timing when a dominant strain was replaced by another strain.

To investigate the dynamics of influenza epidemics and virus evolution, we are now developing a computer simulation that takes infection network, host immunity, and viral evolution into account. A large number of parallel simulations may allow us to infer parameters of the virus evolution under immunological pressure of human population by fitting simulated evolution and actual evolution of the virus. We believe that the simulation could be useful for the more accurate prediction of influenza virus evolution.

2. Exploration of potential zoonotic pathogens by next-generation sequencers

In order to take preemptive measures against zoonoses, a prerequisite is to identify potential pathogens maintained in the wildlife. Recent advances in next-generation sequencing technologies allow us to obtain metagenomic information of samples collected by global surveillance. Given that the volume of sequence data to be analyzed is dramatically increasing, we are constructing a system that utilizes these metagenomic data for the comprehensive exploration of potential zoonotic pathogens.

Toward the control of viral zoonoses
"Influenza virus and filovirus"

Ayato Takada
Division of Global Epidemiology,
Research Center for Zoonosis Control, Hokkaido University

Influenza A viruses of 16 hemagglutinin (HA; H1-H16) and 9 neuraminidase (NA; N1-N9) subtypes are maintained in aquatic birds. Of these, H1N1, H2N2, and H3N2 viruses caused pandemics in humans in the last century, whereas direct avian-to-human transmission of H5N1, H7N7, and H9N2 avian influenza viruses has been frequently reported with a public health concern that a new global pandemic could be caused by these avian-derived viruses, which are antigenically different from the H1, H2, and H3 subtypes.

Ebola and Marburg viruses, members of the filovirus family, cause severe hemorrhagic fever in human and nonhuman primates. No effective filovirus-specific prophylaxis or treatment is yet commercially available. Filovirus species vary genetically, with one in the Marburg virus group and five in the Ebola virus group. Epidemiological efforts to prevent outbreaks lie mainly in identifying natural animal reservoirs. Increasingly frequent outbreaks in Africa and concerns about bioterrorism and imported cases in nonendemic areas point to the importance of public health in two ways – finding strategies to control disease outbreak and developing effective vaccines and drugs.

In this symposium, our recent 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. The goal of the present study is to clarify the relationships between wildlife ecology and zoonosis infection in nature. The recent findings of the study are described as following.

1. Study on Lyme disease prevalence in rodents in 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. burgdorfei*, 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. In 2010 and 2011, we trapped rodents by Sherman traps and pit-hole traps, and collected ticks by flagging in two areas of Hokkaido (central and eastern). Rodent urinary bladders and whole-tick homogenates are being tested for *Borrelia* spp. FlaB gene by PCR, and positive results are being followed up with sequencing. Four *Borrelia* spp. have been, thus far, isolated from rodents: *B. garinii, B. afzelii, B. miyamotoi,* and *B. japonica.* We will present comparisons and relationships between infection rates and species, age, month, and locations of 296 rodents from 7 species trapped in 2010 and the PCR results for 755 rodents from 8 species trapped in 2011 along with locational differences in tick infection rates with *Borrelia* spp.

2. Epidemiological study on tuberculosis in elephants in Nepal

Tuberculosis is a common zoonotic and communicable disease and if this disease is not controlled in captive elephants, then this disease may spill over to wild elephants and other wildlife, domestic animals and human being in Nepal. Although some studies have been taken place on elephant tuberculosis, the type of Mycobacteria i.e. human type or bovine type has not been identified yet. This year we first had a study trip to Nepal and epidemiological search on elephant tuberculosis in Chitwan NP. With a purpose of detection of Mycobacterium tuberculosis complex organism in the trunk discharge and milk samples of captive Asian elephants of Nepal using DNA extraction techniques, we collected samples from 28 elephants but no positive Mycobacterium DNA is detected so far.

3. Epidemiological study on tuberculosis in sika deer in Hokkaido

We presented a paper on a case of tuberculosis in captive sika deer in Osaka last year and start a survey of tuberculosis in sika deer of Hokkaido but no positive deer for tuberculosis have been detected yet.

Studies on diagnoses and pathogeneses of hantavirus infection and leptospirosis

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. Although no hantavirus infection has so far been reported in Japan, attention should be paid to the invasion of hantavirus infection from endemic countries. Leptospirosis is also an important zoonosis of which major reservoir animal is rodent. Since the clinical features of HFRS and leptospirosis are similar and rodents are common reservoir, differential diagnoses is necessary at the areas where hantavirus and leptospira are co-circulating. Therefore, through GCOE program, we have studied about diagnosis of hantavirus infection and leptospirosis, and pathogenicity of the causative agents as follows.

1. The studies on development of immunochromatographic (ICG) test for the detection of anti hantavirus antibody in the rat and human serum have been carried out mainly by the Ms Takako Amada of a master course student. This ICG test uses recombinant nucleocapsid protein expressed by *E. coli* as antigen. Colloidal gold labeled with anti rat IgG antibody and Protein A were applied for rat sera and human sera, respectively. Sensitivity and specificity of ICG test are equal or even higher than IFA and ELISA both in rat and human sera. Furthermore, the ICG test able to serotype human sera obtained from patient of Seoul, Puumala and Sin Nombre type hantavirus infection.

2. The studies about transmission route and pathogeneses of Seoul type hantavirus infection among urban rats population in Vietnam have been carried out mainly by the Dr. Shumpei Yasuda as a GCOE postdoctoral fellow and will be presented in detail at the GCOE program postdoctoral fellow progress report FY2011 presentation.

3. The studies about pathogenicity of *Leptospira* spp by using hamster model and development of serodiagnostic antigens in ELISA have been carried out mainly by the Dr. Chandika Damesh Gamage as a GCOE postdoctoral fellow and will be presented in detail at the GCOE program postdoctoral fellow progress report FY2011 presentation.

In this symposium, recent progress in these subjects will be presented.

Epidemiological study of avian infectious diseases

Kazuhiko Ohashi
Division of Epidemiological research,
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 survey methods using feather tips of birds will be developed to conduct the survey of these infectious diseases in the Hokkaido area. A total of 100 samples were analyzed, but no positive birds of JEV and WNV in the survey of 2011 in the Hokkaido area. In addition, in a joint project with Institute of Environmental Sciences, Hokkaido Research Organization, we also carried out epidemiological surveillance of other viruses, such as Influenza virus, Newcastle disease virus and Marek's disease virus, using either cloacal swabs or feather tips from the wild birds (crows and waterfowls), and isolated Influenza viruses and Newcastle disease viruses but not Marek's disease virus.

The poultry red mite, *Dermanyssus gallinae*, distributed worldwide, is an economically important ectoparasite 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, to search for either good candidates for vaccine or targets for acaricide. Thus, analysis of expressed sequence tags (ESTs) has been performed. A plasmid cDNA library was constructed from the red mite collected from a poultry farm. A total of 2,466 cDNA clones were randomly picked and 1,147 cDNA clones except for shorter inserts were sequenced. These sequences were compared to those accumulated in NCBI databases, 373 sequences were identified as those known of functions. Some of these clones showed high similarity to drug-metabolizing enzymes and vaccine candidates. Among these molecules, homologues of peroxiredoxins (Prx4 and Prx2) and type II allergen, which are suggested as possible vaccine candidates for other ticks were selected, and their immunogenicities were analyzed by using recombinant proteins. Sera from chickens infested with the red mite recognized recombinant Prx4 and type II allergen, showing that these are exposed antigen of the mite. Currently, their functions are being analyzed, and search for new vaccine candidates and target molecules for the clarification of acaricide-resistant mechanism are in progress.

Studies on clinical aspects of human bocavirus, KI polyomavirus and WU polyomavirus infection

Tadashi Ariga
Department of Pediatrics,
Graduate School of Medicine, Hokkaido University

1. Human Bocavirus

Human bocavirus (HBoV, lately denoted HBoV1) has been detected world-widely in 1.6% to 19% of patients with respiratory tract infections. In 2009–2010, three additional species of human bocaviruses, HBoV2, HBoV3 and HBoV4, were discovered from fecal samples. In contrast to HBoV1, HBoV2-4 were predominantly detected in human stool samples and were therefore thought to be involved in enteropathogenesis. We identified HBoV2 (0.6%), HBoV3 (0.4%) and HBoV4 (0.6%), as well as HBoV1 (15.5%), in nasopharyngeal swab samples collected from patients with respiratory tract infections.

2. KI polyomavirus and WU polyomavirus

Polyomaviruses KI (KIPyV) and WU (WUPyV) were detected from 7 (3.0%) and 38 (16.4%) of 232 children with respiratory tract infections by real-time PCR. The rates of infection by KIPyV and WUPyV alone were 3 of 7 (42.9%) and 20 of 38 (52.6%), respectively. In the other samples, various viruses (human respiratory syncytial virus, human metapneumovirus, human rhinovirus, parainfluenza virus 1 and human bocavirus) were detected simultaneously. One case was positive for KIPyV, WUPyV and hMPV. There was no obvious difference in clinical symptoms between KIPyV-positive and WUPyV-positive patients with or without coinfection. KIPyV was detected in one of 30 specimens of lung tissue (3.3%). Neither of the viruses was detected in 30 samples of lung adenocarcinoma tissue.

3. Recent project: Influenza virus

In 2011/12 season, we started the prospective observational study on clinical effects of zanamivir vs. laninamivir against influenza virus infection. Nineteen pediatric outpatient clinics and eleven pediatric hospitals were participated in this project. At present (Feb 26), 820 patients were enrolled in this study. Drug-resistance mutations in the neuraminidase gene will be screened.

Metagenomic approach to identify tick-borne pathogens by using ultra high throughput DNA sequencing and data analyzing technologies

Chihiro Sugimoto
Division of Collaboration and Education
Research Center for Zoonosis Control, Hokkaido University

1) Genone resequencing of *Theileria parva* strains

Theileria parva causes a fatal acute lymphoproliferative disease in cattle named East Coast fever or corridor disease in eastern and southern Africa. The parasite has haploid genome during the most of its life cycle, but a brief diploid stage occurs in ticks that may involve meiotic recombination to increase genetic diversity. In addition, African buffalo serves as a reservoir of this species which can be transmitted to cattle, which makes epidemiology of theileriosis more complex.

Whole genome resequencing of 15 *T. parva* strains including two vaccine strains, field isolates from Zambia and eastern African countires, and buffalo-derived strains was performed by using Illumina Genome Analyzer (Solexa). After mapping Solexa tag sequence onto the reference genome sequence (*T. parva* Muguga atrain), single nucleotide polymorphisms (SNPs) of each strain or clone were identified, which provides genome-wide high-resolution genotyping markers. These results demonstrated that frequent genetic recombination had occurred among strains during transmission through ticks.

2) Metagenomic analysis of tick micobes

Tick can transmit a variety of viral, bacterial and protozoal pathogens, which are often zoonotic. The fact that there are up to 9 rickettsial diseases caused by spotted fever rickettsia newly reported between 1984 and 2001 indicates there are many potential pathogens which have not been unidentified or found in ticks. Metagenomic approach was used to analyze microbial populations of tick microbial floras.

Tick microbes were analysed by two different methods. Firstly, sequence reads from bacteria-enriched fractions analyzed by using Batch Learning Self-organizing Map (BLSOM) prepared form the lysates of 6 tick species. Secondly, amplicon of bacterial small subunit rRNA were analyzed and serched for BLAST aganist bacterial rDNA database.

Both methods identified a variety of bacterial genera and bacterial population profiles were different beween species, male and female in the same species, collections from differnt geographical areas. These results help us to construct a database of tick microbes which may contain unknown zoonotic pathogens. Our efforts to develop the tick pathogen database may lead to the empowerment to predict emerging tick-borne diseases.

Epidemiological study of hantavirus infection in Japan and characterization of tick-borne encephalitis virus isolated in Hokkaido

Hiroaki Kariwa
Laboratory of Public Health, Department of Environmental Veterinary Sciences,
Graduate School of Veerinary Medicine, Hokkaido University

Hantaviruses are rodent-borne zoonotic agents which cause hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS). To understand the recent epidemiology of hantavirus infection in Japanese rodents, 1658 rodents were captured in various regions of Japan from 1994 to 2011. All of the 840 Rattus spp. and 113 wild rodents captured in southern regions of Japan excluding Hokkaido were seronegative. In contrast, among 705 wild rodents and Soricomorpha species from Hokkaido, 7.67% (27/352) of Myodes rufocanus and 1.19% (2/168) of Apodemus speciosus had anti-Hokkaido virus (HOKV) antibodies. These results strongly suggest that the prevalence of hantavirus infection in wild rodents is extremely low in the southern regions of Japan. However, HOKV infection is prevalent in M. rufocanus in Hokkaido. Hantavirus sequences derived from M. rufocanus in Hokkaido, Sakhalin, and Khabarovsk were determined to obtain the insight of evolutionary history of the viruses in these regions. Phylogenetic analyses of viral S and M genes indicated that the virus sequences were separated into 3 different groups depending on the geographical origins.

Tick-borne encephalitis virus (TBEV) causes severe encephalitis to human. Recent serological surveys clarified that TBEV has been endemic in the southern part of Hokkaido over the last 10 years. In this study, we attempted to assess the risk of TBE in the region by analyzing the biological characteristics of the TBEV Oshima 08-AS strain isolated in Hokuto city in 2008. Complete nucleotide sequence (11,100 nucleotides) of Oshima 08-AS was determined. The Oshima 08-AS strain had 36 nucleotide differences resulting in 12 amino acid changes from the Oshima 5-10 strain isolated in 1995. All amino acid changes were located in the region coding for the non-structural (NS) proteins. In virus-infected cells, there is no significant difference between virus multiplication of Oshima 08-AS and Oshima 5-10. In a mouse model, Oshima 08-AS showed higher morbidity and mortality than Oshima 5-10. Relatively higher level of virus was detected in the brains of mice inoculated with Oshima 08-AS. In histopathological analysis, early inflammatory response with the production of viral antigen in neural cells was observed in the brains of the mice infected with Oshima 08-AS.

.

Study on the mechanism of anti-flavivirus infection of oligoadenylate synthetase (OAS)

Takashi Agui
Laboratory of Laboratory Animal Science and Medicine, Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University

The OAS is an anti-flavivirus factor induced by type I interferon. Most of the laboratory mice are susceptible to flavivirus infection including West Nile virus (WNV) due to a nonsense mutation of the *Oas1b* gene. The anti-flavivirus activity of the Oas families is 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 show that mouse Oas1b does not possess enzymatic activity to produce oligo adenylates. Thus, we attempt to elucidate the mechanism of anti-flavivirus activity of mouse Oas1b and OAS from other species.

1. Mouse Oas1b to WNV replicon in BHK cells

Using WNV replicon in BHK cells, B6 type of Oas1b, in which nonsense mutation is present, is surprisingly shown to inhibit the replication of 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 anti-viral 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. Mouse Oas1b to tick-borne encephalitis virus (TBEV) replicon in mouse neuroblastoma cells

Anti-viral activity of B6 and MSM types of Oas1b was assayed using mouse neuroblastoma cells and TBEV replicon. Unlike the results from the WNV replicon, B6 type of Oas1b did not inhibit the replication of TBEV replicon, although MSM type of Oas1b did.

3. Chicken OAS to WNV replicon in BHK cells

Chicken OAS gene was cloned and analyzed its anti-flavivirus activity using WNV replicon in BHK cells. Chicken OAS showed anti-viral activity as well as mouse Oas1b. To elucidate the relationship between anti-viral and enzymatic activities, site-directed mutations were induced in chicken *OAS* gene so as to loose enzymatic activity, and their antiviral activity was assessed. The result was that the replication of WNV replicon was inhibited by all mutant genes as well as the intact gene.

Discussion

Controversial results were obtained by using different virus replicons and host cells. Although it is difficult for us to conclude it at present, the difference might be attributed to the characteristics of the host cells.

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

Ken Katakura
Laboratory of Parasitology, Department of Disease Control
Graduate School of Veterinary Medicine, Hokkaido University

1. Phylogenetic analysis of *Theileria orientalis* in Myanmar based on the *MPSP* gene

Theileria orientalis is a causative agent of bovine benign theileriosis. Currently, *T. orientalis* is classified into 11 genotypes based on sequences of the *major piroplasm surface protein* (*MPSP*) gene. Previous study showed that 257 of 521 (49.3%) cattle blood DNA samples were positive for PCR of the *MPSP* gene. In the present study, 54 of 257 *MPSP* gene fragments were sequenced, followed by the phylogenetic analysis. As a result, six genotypes (Types 1, 3, 4, 5 and 7, and Type N-3) were determined, indicating that *T. orientalis* circulating in Myanmarese cattle have a higher genetic diversity as reported in the neighboring countries such as Thailand and Vietnam.

2. Characterization of *F. gigantica* and *Fasciola* sp. in cattle and buffaloes in Myanmar

In Asian countries, liver flukes of spermatogenic (sexual reproductive) *Fasciola gigantica* and aspermic (parthenogenetic) *Fasciola* sp. are mainly distributed. Among 88 liver flukes collected from cattle and water buffaloes in Myanmar, 8 were aspemic flukes with the mitochondrial *nad1* haplotype that is also found in China, Vietnam, Korea and Japan. Meanwhile, 80 were *F. gigantica* with different 17 *nad1* haplotypes unique to Myanmar, suggesting of a unique history of the parasite introduction into Myanmar.

3. Mechanisms of the persistence of *Leishmania donovani* parasites in immunodeficient alymphoplasia *aly/aly* mice

Previous study using *aly/aly* mice with a mutation of NF-κB inducing kinase gene as an immunodeficient visceral leishmaniasis model suggested that $CD4^+Foxp3^+$ Tregs play a significant role in the persistence of *L. donovani* especially in the liver. In the present study, immunohistochemical analysis revealed that $Foxp3^+$ T cells were adjacent to $CD3^+Foxp3^-$ cells (probably including effector T cells) and parasite-containing phagocytic cells in the chronic hepatic granulomas. By laser microdissection and quantitative RT-PCR analysis of the granulomas, although both *TGF-β* and *IL-10* mRNAs increased after infection, the expression levels of *IL-10* mRNA were more correlated with those of *Foxp3*, suggesting that IL-10 may be involved in the suppressive function of Tregs.

Epidemiological investigations of leptospirosis and rabies in Sri Lanka in 2011

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

I will present our research activities on leptospirosis and rabies in Sri Lanka and teaching program on Epidemiological Methods in 2011.

Leptospirosis:
A cross sectional survey was conducted among community people above 15 years of age in some selected areas in Kegalle district in Sabaragamuwa Province of Sri Lanka from Dec. 2011 to Jan. 2012. Target people were asked to participate in a 15-20 minutes face-to-face interview on their knowledge, attitudes and practices (KAP) to the disease. Nine-hundred-thirteen (913) people agreed to participate in the survey. The questionnaire consisted of one's demographics information and daily life style in addition to KAP. The data were collected upon the receipt of informed consent from each participant and the ethical approval for the survey was obtained from the Ethical Committee of Medical School of the University of Peradeniya (UP).

Preliminary results indicated that the respondents were female-dominant (64%). Among all, 98% knew leptospirosis as "rat fever" in their local language and they recognized rat as the major reservoir animal. On the contrary, less than 1/3 of them knew that buffalos and dogs were also reservoir animals for leptospirosis. More results will be presented in the meeting (Refer to Dr Obayashi's abstract).

Rabies:
We have collected about 300 dog sera together with some epidemiological information in 3 cities of the Western Province in collaboration with Public Health Veterinary Service and Dept. of Veterinary Pathobiology, UP. Some preliminary data will be presented in the meeting (Refer to Ms Shiokawa's abstract). In addition, some activities having been carried out in the Rabies Control Unit will be discussed.

Teaching:
Two-week intensive course on Epidemiological Methods was held in August 2011 with a good participation of GCOE trainees.

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. However, how prions propagate in cells, how microglia and astrocytes recognize and respond to prion propagation, and the mechanism of neuronal death by prion propagation remain to be elucidated. To understand the pathogenesis of prion infection, this year, we attempted to clarify 1) early event of prion infection in cells, 2) temporal order of astrocyte microglial activation in brains of prion-infected mice, and 3) influence of prion infection in differentiated mouse neurospheres.

To analyze the events in the early stage of prion infection in cells, intracellular dynamics of fluorescent dye-labeled-PrP^{Sc} and newly generated PrP^{Sc} was monitored. Most of inoculated PrP^{Sc} was co-localized with low density lipoprotein, a marker of the endo-lysosomal pathway, but not with transferrin, a marker of the endocytic recycling pathway. In contrast to the inoculated PrP^{Sc}, the newly generated PrP^{Sc} was localized at peri-nuclear regions of the cell, possibly endocytic recycling compartments (ERC). Furthermore, overexpression of a dominant negative mutant of Rab7 and wild-type Rab9, which are known to inhibit the trafficking from early to late endosomes and from late endosomes to peri-nuclear organelle such as TGN and ERC, respectively, decreased the generation of PrP^{Sc}. These results suggest that establishment of prion infection strongly associates with intracellular membrane traffic from the endo-lysosomal to the endocytic recycling pathway.

Distinction of PrP^{C} from PrP^{Sc} in the immunohistochemical staining is one of technical difficulties in the analysis of tissues from prion-infected animals. Recently we reported that mAb 132, recognizing the aa 119-127 of PrP molecule, is tremendously useful for specific detection of PrP^{Sc} in both prion-infected cells and tissues. Using this PrP^{Sc}-specific detection, we found that astrocyte activation precedes microglial activation in the early stage of infections. Thus, astrocytes may recognize prion propagation directly or subtle changes in neurons by prion propagation and play an important role in neuropathobiology in the early stage of prion infection.

The third topic, the characterization of prion-infected neurospeheres, will be presented by Dr. Yukiko Sassa, PD of GCOE program, in the annual report by PD and RA.

Investigation of viral assembly mechanism of Polyomavirus and molecular epidemiological research of rabies in Zambia

Hirofumi Sawa
Division of Molecular Pathobiology
Research Center for Zoonosis Control, Hokkaido University

Part I: Molecular biological investigation of viral assembly mechanism of Polyomavirus.

Polyomavirus capsid shell consists of 72 capsomeres of a major structural protein Vp1. Five VP1 monomers interdigitate their secondary structures to form pentameric capsomeres. Previous studies of SV40 Polyomavirus have suggested that cysteine residues participate in the formation of Vp1 pentamers and virion assembly. However, it remains to be elucidated whether the formation of human Polyomavirus, JC virus (JCV)-Vp1 pentamer and its assembly also requires cysteine residues. In the current study, we have tested the hypothesis that JCV-Vp1 cysteine residues, including C42, C80, C97, C200, C247, and C260, play a role in folding and oligomerization of JCV-Vp1. We found that a Vp1 pentamer can form *via* S-S linkages, perhaps, through many of 6 cysteines. Furthermore, analysis of many multiple cysteine mutants suggested that a region of Vp1 including C80 is sensed for proper folding by many host factors (This work was mainly achieved by Dr. Shintaro Kobayshi.).

Part II: Molecular epidemiological research of rabies virus in Zambia.

The lineage of rabies virus (RABV) in Zambia was determined by phylogenetic analysis of the nucleoprotein (N) and glycoprotein (G) gene sequences. Total RNA was extracted from 87 direct fluorescent antibody test brain specimens out of which only 35 (40%) were positive on nested RT-PCR for each gene, and 26 being positive for both genes. Positive specimens for the N (n=33) and G (n=35) gene were used for phylogenetic analysis. Phylogenetic analysis of the N gene showed two phylogenetic clusters in Zambia belonging to the Africa 1b lineage present in eastern and southern Africa. While one cluster exclusively comprised Zambian strains, the other was more heterogeneous regarding the RABV origins and included strains from Tanzania, Mozambique, and Zambia. Phylogenetic analysis of the G gene revealed similar RABV strains in different hosts and regions of Zambia. We designed primers for RT-LAMP assay from the consensus sequence of the N gene in an attempt to improve the molecular diagnosis of RABV in Zambia. The specificity and reproducibility of the RT-LAMP assay was confirmed with actual clinical specimens. Therefore, the RT-LAMP assay presented in this study may prove to be useful for routine diagnosis of rabies in Zambia (This work was mainly achieved by Dr. Muleya Walter.).

Successful treatment of rabid rabbits by intrathecal immunization

Takashi Umemura
Laboratory of Comparative Pathology,
Graduate School of Veterinay Mecicine, Hokkaido University

Background

Our previous studies have demonstrated that intrathecal immunization (IT) induced antigen-specific antibodies not only in the serum but also in the cerebrospinal fluid (CSF), and IT-immunized mice were completely protected against pseudorabies and rabies virus challenges in the peripheral tissue. Furthermore, 80% of IT-immunized mice tolerated intracerebral rabies virus challenge without any clinical sighs. Antigen-specific antibodies induced in the CSF were originated both from the antibodies of peripheral blood and de novo antibody locally produced in the central nervous system (CNS).

Results of experiment of this year

Thirteen female New Zealand white rabbits were allotted into 4 groups. In group 1, 3 rabbits were inoculated with rabies virus (CVS strain) into hind legs. In group 2, 3 rabbits received subcutaneous (SC) vaccination using inactivated rabies virus vaccine (Nisseiken Co.). Three days after the vaccination, the rabbits were challenged with the rabies virus in the hind legs. In group 3, 3 rabbits received SC vaccination and rabies virus challenge as group 2. After the rabbits show clinical signs of rabies, the rabbits were treated with SC immunization 4 times on 0, 1, 3 and 5 days after initiation of clinical signs. In group 4, 4 rabbits were SC immunized and challenged with rabies virus in the same manner as group 2. After confirming the clinical signs, the rabbits were treated with IT immunization 4 times on 0, 1st, 3rd, 5th and 9th day of clinical signs.

All the rabbits of groups 1 and 3, and 3 of 4 rabbits of group 2 showed hind leg paralysis 5-8 days after virus inoculation and stopped eating and drinking. The nervous signs gradually progressed to systemic convulsion thereafter and died within 4 to 7 days after initiation of the clinical signs. All the rabbits in group 4 and one rabbit in group 2 also showed the nervous signs and progressed to systemic convulsion on the same time course. However, these rabbits gradually started eating and drinking from 5 to 7 days after showing the nervous signs and survived until termination of experimental period (35 days after virus inoculation). Histopathologically, a massive amount of virus antigen was found in nerve cells throughout the CNS and dorsal ganglions of died rabbits. Whereas, the viral antigen was not found or minimal in the rabbits survived and sacrificed at the end of the experiment.

Host cell factors involved in synthesis and expression of borna disease virus and avian bornavirus glycoproteins

Yayoi Otsuka and Mutsumi Inaba
Laboratory of Molecular Medicine
Graduate School of Veterinary Medicine, Hokkaido University

< Background & Aim >

Borna disease virus (BDV) glycoprotein (BDV-G) expression is extremely low in the cells persistently infected with BDV and in the cells transfected with BDV-G cDNA. We previously reported that the expression of avian bornavirus glycoprotein (ABV-G) was remarkably higher than that of BDV-G in transfected cells, despite the similarities in their polypeptide backbone. We also demonstrated that the amino acid sequence of the transmembrane domain affected the plasma membrane expression level of BDV-G. However, the mechanism for this phenomenon remains unknown. The purpose of the present study was to unravel the mechanism for the restricted expression of BDV-G.

< Results >

1) The open reading frame of the BDV-G gene contains the splicing elements consisting of the 5'-splice donor site (SD), branch point, polypyrimidine tract (PPT), and 3'-splice acceptor site (SA), while that of the ABV-G gene lacks the nucleotide sequence corresponding to the SD. Northern blotting and immunoblotting analyses of the cells transfected with a series of BDV-G and ABV-G mutants revealed that the splicing event was the primary cause for the low level of BDV-G expression and suggested that the PPT sequence preceding the SA in the BDV-G gene was more efficient in splicing than that in the ABV-G gene. Interestingly, however, overexpression of a cellular splicing factor U2AF65 that binds to PPT sequences caused an increase in BDV-G expression. These findings suppose some modulatory roles of cellular splicing factors including U2AF65 in the expression of BDV-G.

2) The FLAG-tagged BDV-G bearing the artificially modified PPT sequences was expressed in 293T cells and its transport from the ER to the plasma membrane was analysed. The immunoprecipitation of BDV-G and ABV-G co-precipitated the ER chaperons, calnexin and BiP. Overexpression of Sar1-GTP, but not that of ARF1-GTP, resulted in markedly reduced expression of the furin-cleaved forms of these glycoproteins at the cell surface. The vast majority of *N*-glycans on cell-surface BDV-G and ABV-G was sensitive to endoglycosidase H, indicating insufficient *N*-glycan processing in the Golgi.

< Conclusion >

Expression of BDV-G in the transfected cells is restricted by the cellular splicing event. Once synthesized, BDV-G and ABV-G appear to be transported to the cell surface through the conventional secretory pathway.

Molecular characterization of drug resistant *Mycobacterium tuberculosis* from Asian countries

Yasuhiko Suzuki
Division of Global Epidemiology,
Hokkaido University Research Center for Zoonosis Control

<Introduction> Multidrug-resistant *Mycobacterium tuberculosis* (MDR-TB) has become a major problem in tuberculosis treatment. Prompt determination of MDR-TB is important not only for the appropriate treatment of patients but also for the prevention of the spread of resistant tubercle bacilli. In *M. tuberculosis*, resistance against rifampicin and isoniazid is highly associated with mutations on the responsible genes, i.e., *rpoB* for rifampicin, *katG* and *inhA* for isoniazid. Recent technology makes it possible to determine MDR-TB quickly by checking specific mutations using PCR and hybridization. However, the ratio of each mutation has not been determined in many countries despite it can differ in each country. In this study, we determined mutations that possibly related to drug resistance in MDR-TB isolates obtained in Bangladesh, Nepal, Myanmar and Japan.

<Samples and Methods> MDR-TB isolates collected in Bangladesh (n=220), Nepal (n=47), Myanmar (n=132) and Japan (n=89) were used for the mutation analysis. Following gene regions were sequenced and analyzed, *rpoB*: nucleotide position 1276-1356 (rifampicin resistance determining region, RRDR), *katG*: 823-1140, *inhA*: minus50 - minus1 (promoter region).
<Results> In *rpoB* RRDR, most frequently observed mutation was C 1349 T in all countries and its ratio among MDR-TB was around 50% (40-64%). Most of other mutations were also commonly found in all country samples and sum of the ratios of top seven prevalent mutations were more than 70% in each group. The percentage of MDR-TB isolates with no mutations in *rpoB* RRDR was 4.5% (3.4-6.4%). Regarding isoniazid resistance, G 944 C mutation in *katG* and C -15 T mutation in *inhA* were majorities among observed mutations and the sum ratios of these among MDR-TB were 87, 89, 94 and 48% in Bangladesh, Nepal, Myanmar and Japan, respectively. No mutations were observed in either *katG* or *inhA* in 5.9, 4.3 and 2.3% in Bangladesh, Nepal and Myanmar MDR-TB isolates, respectively, whereas the ratio was 34.8% in Japanese isolates.

<Conclusions> The variation and ratio of mutations on *rpoB, katG* or *inhA*, which are thought to be associated with rifampicin or isoniazid resistance, were similar among MDR-TB isolates in Bangladesh, Nepal, Myanmar and Japan. More than 70% of rifampicin resistant isolates can be determined in these countries by detecting seven types of *rpoB* mutations. For isoniazid resistance prediction, most of the resistant isolates can be determined by detecting two major mutations in *katG* and *inhA* in Bangladesh, Nepal and Myanmar. However, additional analyses will be needed for the determination of isoniazid resistant TB in Japan because of the higher ratio of resistant isolates that were not relying on those two mutations. Thus, surveillance of resistance-associated mutations in each country seems to be important for an effective determination of drug resistant *M. tuberculosis* by rapid mutation detection systems with PCR and hybridization.

Regulation of the Akt kinase by TCL1 family oncogene TCL1b; implications for developing pharmacological reagents for Akt

Masayuki Noguchi
Division of Cancer Biology, Institute for Genetic Medicine, Hokkaido University

In recent years, pandemic influenza of H1N1 avian influenza viruses have emerged and now appear to spread in many regions throughout the world. Among the 11 influenza viral proteins, NS1 (non-structural protein) has been implicated in the regulation of several biological functions, including regulation of apoptosis suppression of host immune responses, inhibition of nuclear export of mRNA and splicing of mRNA. We found that influenza virus products NS1 interacted preferentially with phosphorylated Akt and enhanced Akt kinase activity, suggesting that Akt-NS1 as a putative target for the therapy of influenza virus infection.

Previously, we have identified "*Akt-in*", a novel small peptide inhibitor (Akt inhibitor, NH2-AVTDHPDRLWAWEKF-COOH) based on the structural functional study of Akt-TCL1 protein complexes. The ptotooncogene TCL1 families (TCL1, TCL1b, and MTCP1) share a relatively high degree of amino acid homology (30-50%) and a unique, symmetrical β-barrel structure. In part of the effort to develop better inhibitory activity of"*Akt-in*" by the structural-functional analysis of TCL1b, we examined whether and how TCL1b might have similar function as Akt kinase co-activator.

1. In co-immunoprecipitation assays, TCL1b co-immunoprecipitated with Akt.
2. In vitro Akt kinase assays, TCL1b enhanced Akt kinase activity.
3. By Agilent Expression Array analysis, TCL1b showed highly significant homologous gene induction pattern as myr-Akt, a constitutive active form of Akt or TCL1 in cluster analysis, KEGG pathway, and Gene Ontology.
4. Colony transformation assay demonstrated TCL1b exhibited stronger potency of oncogenicity than TCL1 or myr-Akt.
5. CMV enhancer -β-actin promoter driven TCL1b transgenic mice exhibited hemangiosarcoma from intestinal origin.
6. Human cancer tissue array analysis showed TCL1B stained positive 68/146 cases.

These observations suggested that analogous to TCL1, TCL1b also functions as an Akt kinase co-activator and possibly plays an active role in oncogenicity in human neoplastic diseases. Based on this findings, we are currently aiming to develop newer version of Akt inhibitor "*Akt-in*" based on the structure of Akt-TCL1b protein complexes for inhibiting influenza virus replication by down-modulating the NS1-Akt functional interaction.

Functional role of the extracellular matrix to determine the severity of influenza

Yosuke Nakayama, Tadaaki Miyazaki
Research Section of Probiotics Immunology,
Institute for Genetic Medicine, Hokkaido University

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.

One of the ECM proteins, osteopontin (OPN), a glycosylated phosphoprotein, is involved in exacerbating various inflammatory diseases. A severe pulmonary inflammation is frequently found in lethal influenza A virus (IAV) infection. However, the function of OPN against the infection was poorly understood. Here, we demonstrate an importance of OPN on immune response and disease severity after IAV infection. We found that the expression level of OPN was increased in mice infected with IAV. The OPN knockout (KO) mice exhibited a severe pathological phenotype and the survival rate decreased after the lethal IAV infection, compared to the wild type mice, while the survival rate increased in OPN transgenic (Tg) mice. The population of natural killer (NK) cells significantly decreased in OPN KO mice at day 5 after the infection, whereas, it increased in OPN Tg mice. These results suggest that OPN plays an important role in host defense against IAV infection through the regulation of NK cell population.

The degradation of sturdy structural ECM proteins is catalyzed by matrix metalloproteinases (MMPs). MMPs and their inhibitors play an important regulatory role in the inflammatory responses and physiologic tissue remodeling. However, the functional role of these molecules (ECMs, cell adhesionmolecules, MMPs and MMP inhibitors) after IAV infection is still unclear. We found alterationsof gene expression of several MMPs and ECMs in the lungs of mice infected with IAV. In particular, the mRNA expression of tissue inhibitor of metalloproteinase-1 (TIMP-1), which is aspecific inhibitor of MMPs, was remarkably induced in the lungs after IAV infection. Therefore,we focused on the function of TIMP-1 for the pathogenesis, and infected TIMP-1 KO andwild type (WT) mice with the virus. After IAV infection, the survival rate of TIMP-1 KO miceas lower than that of WT mice. In addition, at 7 day after the infection, severe hemorrhagic tendency was detected in the bronchoalveolar lavage fluid (BALF) in TIMP-1 KO mice, and inflammatory cell accumulation in BALF increased in TIMP-1 KO mice, compared with WT mice. Therefore, it is suggested that TIMP-1 is involved in the regulation of vascular permeability in the lung inflammatory legion after IAV infection, and protects lung tissue from excessive inflammatory response such as hyperemia, exudation, and edema after the infection.

Molecular-based study for the control of *Bacillus anthracis* infection

Hideaki Higashi
Division of Infection and Immunity,
Research Center for Zoonosis Control, Hokkaido University

Bacillus anthracis is a Gram-positive spore-forming bacterium that persists in soil for long periods of time. *B. anthracis* infects its host by the entry of spores through skin abrasions, the ingestion of contaminated food or via the respiratory system and the etiological agent of anthrax. Anthrax is a disease of wildlife, livestock and human that remains a public health problem in the world. In endemic areas, control of livestock disease through vaccination and surveillance is essential for preventing human anthrax. However, sufficient surveillance in the area is scarcely done and vaccination of every animal is not feasible. Furthermore, safe and effective vaccine for human is not available to the general public. With a problem of bacterial resistance to antibiotics, it is necessary to improve our understanding of *B. anthracis* through surveillances and develop more effective prophylaxis against *B. anthracis* infection. Thus, we start a study of *B. anthracis* to control anthrax in human and animals, and we are trying to develop a vaccine that is based on structural information for *B. anthracis* toxin, PA protein.

・Introduction of molecular diagnostic methods for *B. anthracis* infection to Zambia.

In August and September 2011, about 400 cases of human anthrax were found in eastern area of Zambia with 5 people dying. It was suspected that patients contacted with *B. anthracis* contaminated hippopotamus meat. We urgently introduced molecular diagnostic tests to Zambia and identified *B. anthracis* from meats of the hippopotamus that died an unnatural death and surrounding soil in the outbreak area. The introduced diagnostic methods are transferred to UNZA and utilized for the diagnosis by researchers of UNZA.

・Preparation of materials to develop a vaccine against *B. anthracis.*

To develop a vaccine against *B. anthracis*, we cloned *B. anthracis* protective antigen (PA) gene from Zambian strain that was isolated from the hippopotamus sample at HUCZCZ. Furthermore, we are preparing cell lines that express anthrax toxin receptor (ATR) 1/2 ectopically for establishing toxicity test of *B. anthracis* pathogenic factors (*in vitro* reconstitution assay with PA, lethal factor LF and edema factor EF).

〒060-0818 札幌市北区北18条西9丁目
北海道大学大学院獣医学研究科 111号室 グローバルCOE推進室
Tel/fax 011-706-5294, gcoe@vetmed.hokudai.ac.jp
http://www.vetmed.hokudai.ac.jp/gcoe/