International Research Center for Infectious Diseases

Department of Special Pathogens
高病原性感染症研究部門

Professor Chieko Kai, D.V.M., Ph.D.
Professor Yoshihiro Kawaoka, D.V.M., Ph.D.

教授(兼) 農学博士 甲 斐 知恵子
教授(兼) 獣医学博士 河 岡 義 裕

Highly pathogenic viral agents causing emerging infectious diseases are of concern not only to public health but also as possible biological weapons. The ultimate goal of our research is to unlock the secrets of the pathogenicity of such viruses in humans and to develop effective vaccines and antiviral compounds against these pathogens. We have been investigating the molecular basis of the replication cycle and extreme virulence of special pathogens, using Ebola, influenza, and Nipa viruses as models.

Region of Nipah virus C protein responsible for shuttling between the cytoplasm and nucleus.

Horie, R., Yoneda, M., Uchida, S., Sato, H. and Kai, C.

Nipah virus (NiV) causes severe encephalitis in humans, with high mortality. NiV nonstructural C protein (NiV-C) is essential for its pathogenicity, but its functions are unclear. In this study, we focused on NiV-C trafficking in cells and found that it localizes predominantly in the cytoplasm but partly in the nucleus. An analysis of NiV-C mutants showed that amino acids 2, 21-24 and 110-139 of NiV-C are important for its localization in the cytoplasm. Inhibitor treatment indicates that the nuclear export determinant is not a classical CRM1-dependent nuclear export signal. We also determined that amino acids 60-75 and 72-75 were important for nuclear localization of NiV-C. Furthermore, NiV-C mutants that had lost their capacity for nuclear localization inhibited the interferon (IFN) response more strongly than complete NiV-C. These results indicate that the IFN-antagonist activity of NiV-C occurs in the cytoplasm.

Risk assessment of recent Egyptian H5N1 influenza viruses

Arafa AS[1], Yamada S, Imai M, Watanabe T, Yamayoshi S, Iwatsuki-Horimoto K, Kiso M, Sakai-Tagawa Y, Ito M, Imamura T, Nakajima N[2], Takahashi K[2], Zhao D, Oishi K, Yasuhara A, Macken CA[3], Zhong G[4], Hanson AP[4], Fan S[4], Ping J[4], Hatta M[4], Lopes TJ[4], Suzuki Y[5], El-Husseiny M[1], Selim A[1], Hagag N[1], Soliman M[6], Neumann G[4], Hasegawa H[2], Kawaoka Y.: [1]National Laboratory for Veterinary Quality Control on Poultry Production, Animal Health Research Institute, Dokki, Giza, Egypt. [2]Department of Pathology, National Institute of Infectious Diseases, Sinjuku-ku, Tokyo 162-8640, Japan. [3]Bioinformatics Institute, The University of Auckland, Auckland 1142, New Zealand. [4]Influenza Research Institute, Department of Pathobiological Sciences, School of Veterinary Medicine, University of Wisconsin-Madison, Madison, WI 53711, USA. [5]College of Life and Health Sciences, Chubu University, Aichi 487-8501, Japan. [6]General Organization for Veterinary Services, Dokki, Giza, Egypt.

Highly pathogenic avian influenza (HPAI) viruses of the H5N1 subtype are enzootic in poultry populations in different parts of the world, and have caused numerous human infections in recent years, particularly in Egypt. However, no sustained human-to-human transmission of these viruses has

yet been reported. We tested nine naturally occurring Egyptian H5N1 viruses (isolated in 2014-2015) in ferrets and found that three of them transmitted via respiratory droplets, causing a fatal infection in one of the exposed animals. All isolates were sensitive to neuraminidase inhibitors. However, these viruses were not transmitted via respiratory droplets in three additional transmission experiments in ferrets. Currently, we do not know if the efficiency of transmission is very low or if subtle differences in experimental parameters contributed to these inconsistent results. Nonetheless, our findings heighten concern regarding the pandemic potential of recent Egyptian H5N1 influenza viruses.

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International Research Center for Infectious Diseases

Department of Infectious Disease Control
感染制御系

Professor Yasushi Kawaguchi, D.V.M., Ph.D.

教授（兼） 獣医学博士 川 口 寧

Our special interest is focused upon searching for effective methods to protect or control viral infection by using accumulated knowledge based on molecular pathogenicity, and developing novel anti-viral drugs and attenuated strains for novel vaccines. The works have been conducted by close collaboration with Division of Molecular Virology, Department of Microbiology and Immunology.

1. Cellular Transcriptional Coactivator RanBP10 and Herpes Simplex Virus 1 ICP0 Interact and Synergistically Promote Viral Gene Expression and Replication

Yuka Sato, Akihisa Kato, Yuhei Maruzuru, Masaaki Oyama[1], Hiroko Kozuka-Hata[1], Jun Arii and Yasushi Kawaguchi: [1]Medical Proteomics Laboratory, The Institute of Medical Science, The University of Tokyo, Minato-ku, Tokyo 108-8639, Japan

To investigate the molecular mechanism(s) by which herpes simplex virus 1 (HSV-1) regulatory protein ICP0 promotes viral gene expression and replication, we screened cells overexpressing ICP0 for ICP0-binding host cell proteins. Tandem affinity purification of transiently expressed ICP0 coupled with mass spectrometry-based proteomics technology and subsequent analyses showed that ICP0 interacted with cell protein RanBP10, a known transcriptional coactivator, in HSV-1-infected cells. Knockdown of RanBP10 in infected HEp-2 cells resulted in a phenotype similar to that observed with the ICP0-null mutation, including reduction in viral replication and in the accumulation of viral immediate-early (ICP27), early (ICP8), and late (VP16) mRNAs and proteins. In addition, RanBP10 knockdown or the ICP0-null mutation increased the level of histone H3 association with the promoters of these viral genes, which is known to repress transcription. These effects observed in wild-type HSV-1-infected HEp-2 RanBP10 knockdown cells or those observed in ICP0-null mutant virus-infected control HEp-2 cells were remarkably increased in ICP0-null mutant virus-infected HEp-2 RanBP10 knockdown cells. Our results suggested that ICP0 and RanBP10 redundantly and synergistically promoted viral gene expression by regulating chromatin remodeling of the HSV-1 genome for efficient viral replication.

2. Ubiquitin-Specific Protease 9X in Host Cells Interacts with Herpes Simplex Virus 1 ICP0

Yuka Sato, Akihisa Kato, Jun Arii, Naoto Koyanagi, Hiroko Kozuka-Hata, Masaaki Oyama and Yasushi Kawaguchi

Herpes simplex virus 1 (HSV-1) expresses infected cell protein 0 (ICP0), a multi-functional protein with E3 ubiquitin ligase activity and a critical regulator of the viral life cycle. To obtain novel insights into the molecular mechanism by which ICP0 regulates HSV-1 replication, we analyzed HEp-2 cells infected with HSV-1 by tandem affinity purification and mass spectrometry-based proteomics. This screen identified 50 host-cell proteins that po-

tentially interact with ICP0, including ubiquitin-specific protease 9X (USP9X). The interaction between ICP0 and USP9X was confirmed by co-immunoprecipitation. Notably, USP9X depletion increased the ICP0 abundance and promoted viral replication. These results suggest that USP9X-dependent regulation of ICP0 expression is part of a complex feedback mechanism that facilitates optimal HSV-1 replication.

3. Characterization of a herpes simplex virus 1 (HSV-1) chimera in which the Us3 protein kinase gene is replaced with the HSV-2 Us3 gene

Keiko Shindo, Akihisa Kato, Naoto Koyanagi, Hiroshi Sagara[1], Jun Arii and Yasushi Kawaguchi: [1]Medical Proteomics Laboratory, The Institute of Medical Science, The University of Tokyo, Minato-ku, Tokyo 108-8639, Japan

Us3 protein kinases encoded by herpes simplex virus 1 (HSV-1) and 2 (HSV-2) play important roles in viral replication and pathogenicity. To investigate type-specific differences between HSV-1 Us3 and HSV-2 Us3 in cells infected by viruses with all the same viral gene products except for their Us3 kinase, we constructed and characterized a recombinant HSV-1 in which its Us3 gene was replaced with the HSV-2 Us3 gene. Replacement of HSV-1 Us3 with HSV-2 Us3 had no apparent effect on viral growth in cell cultures or on the range of proteins phosphorylated by Us3. HSV-2 Us3 efficiently compensated for HSV-1 Us3 functions, including blocking apoptosis, controlling infected cell morphology, and down-regulating cell surface expression of viral envelope glycoprotein B. In contrast, replacement of HSV-1 Us3 by HSV-2 Us3 changed the phosphorylation status of UL31 and UL34, which are critical viral regulators of nuclear egress. It also caused aberrant localization of these viral proteins, aberrant accumulation of primary enveloped virions in membranous vesicle structures adjacent to the nuclear membrane, and reduced viral cell-cell spread in cell cultures and pathogenesis in mice. These results clearly demonstrated biological differences between HSV-1 Us3 and HSV-2 Us3, especially in regulation of viral nuclear egress and phosphorylation of viral regulators critical for this process. Our study also suggested that the regulatory role(s) of HSV-1 Us3, which were not carried out by HSV-2 Us3 was important for HSV-1 cell-cell spread and pathogenesis in vivo.

4. Roles of Us8A and its Phosphorylation Mediated by Us3 in Herpes Simplex Virus 1 Pathogenesis

Akihisa Kato, Tomoko Ando, Shinya Oda, Mizuki Watanabe, Naoto Koyanagi, Jun Arii and Yasushi Kawaguchi

The herpes simplex virus 1 (HSV-1) Us8A gene overlaps the gene that encodes for glycoprotein E (gE). Previous studies have investigated the roles of Us8A in HSV-1 infection using null-mutations in Us8A and gE; therefore, the role of Us8A remains to be elucidated. In this study, we investigated the function of Us8A and its phosphorylation at serine 61 (Ser-61), which we recently identified as a phosphorylation site by mass spectrometry-based phosphoproteomic analysis of HSV-1-infected cells, in HSV-1 pathogenesis. We observed that (i) the phosphorylation of Us8A Ser-61 in infected cells was dependent on the activity of the virally encoded Us3 protein kinase; (ii) the Us8A null mutant virus exhibited a 10-fold increase in the 50% lethal dose for virulence in the central nervous system (CNS) of mice following intracranial infection compared with a repaired virus; (iii) replacement of Ser-61 with alanine (S61A) in Us8A had little effect on virulence in the CNS of mice following intracranial infection, whereas it significantly reduced the mortality of mice following ocular infection to levels similar to the Us8A null mutant virus; (iv) the Us8A S61A mutation also significantly reduced viral yields in mice following ocular infection, mainly in the trigeminal ganglia and brains; and (v) a phosphomimetic mutation at Us8A Ser-61 restored wild-type viral yields and virulence. Collectively, these results indicate that Us8A is a novel HSV-1 virulence factor and suggest that the Us3-mediated phosphorylation of Us8A Ser-61 regulates Us8A function for viral invasion into the CNS from peripheral sites.

5. p53 is a Host Cell Regulator during Herpes Simplex Encephalitis

Yuhei Maruzuru, Naoto Koyanagi, Naoki Takemura[1,2], Satoshi Uematsu[1,2], Daisuke Matsubara[3,4], Yutaka Suzuki[5], Jun Arii, Akihisa Kato and Yasushi Kawaguchi: [1]International Research and Development Centre for Mucosal Vaccine, Institute for Medical Science, The University of Tokyo, Tokyo, Japan [2]Department of Mucosal Immunology, School of Medicine, Chiba University, Chiba 260-8670, Japan [3]Molecular Pathology Laboratory, Institute of Medical Science, University of Tokyo, Tokyo, Japan [4]Department of Integrative Pathology, Jichi Medical University, Tochigi, Japan [5]Department of Computational Biology and Medical Sciences, Graduate School of Frontier Sciences, The University of Tokyo, Kashiwa 277-8568, Japan

p53 is a critical host cell factor in the cellular response to a broad range of stress factors. We re-

cently reported that p53 is required for efficient herpes simplex virus 1 (HSV-1) replication in cell culture. However, a defined role for p53 in HSV-1 replication and pathogenesis in vivo remains elusive. In this study, we examined the effects of p53 on HSV-1 infection in vivo using p53-deficient mice. Following intracranial inoculation, p53 knockout reduced viral replication in the brains of mice and led to significantly reduced HSV-1 mortality due to encephalitis. These results suggest that p53 is an important host cell regulator for HSV-1 replication and pathogenesis in the central nervous system (CNS).

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International Research Center for Infectious Diseases

Department of Infectious Disease Control Division of Viral Infection

感染制御部門　ウイルス学分野

Associate Professor　Takeshi Ichinohe, Ph.D.

准教授　工学博士　　一　戸　猛　志

We focus on understanding how viruses are recognized by NLRP3 inflammasome and how the innate recognition receptor controls antigen-specific adaptive immune responses. We study immune responses to influenza viruses in the lung. Our recent focus also includes the study of how microbiota regulates adaptive immune responses to these pathogens. Our ultimate goal is to utilize the knowledge we gain through these areas of research in the rational design of effective vaccines for the prevention of infectious diseases.

1. Influenza A virus NS1 protein inhibits NLRP3 inflammasome-mediated IL-1β secretion

Moriyama M, Chen IY, Kawaguchi A, Koshiba T, Nagata K, Takeyama H, Hasegawa H and Ichinohe T

Innate immune sensing of influenza virus via pattern-recognition receptors not only plays a key role in generating type I interferons but also triggers inflammatory responses. We previously demonstrated that influenza A virus M2 protein activates the NLRP3 inflammasome, leading to the secretion of interleukin 1 beta (IL-1β) and IL-18 following the activation of caspase-1. Although the nonstructural protein 1 (NS1) of influenza virus inhibits IL-1β secretion, the precise mechanism by which it achieves this remains to be defined. Here, we demonstrate that the NS1 protein interacts with NLRP3 to suppress the NLRP3 inflammasome activation. J774A.1 macrophages stably expressing the NS1 protein suppressed NLRP3 inflammasome-mediated IL-1β secretion. The NS1 RNA-binding domain (basic residues 38 and 41) and TRIM25-binding domain (acidic residues 96 and 97) are important for suppression of NLRP3 inflammasome-mediated IL-1β secretion. These results shed light on a mechanism by which the NS1 protein of influenza virus suppresses NLRP3 inflammasome-mediated IL-1β secretion.

2. Consecutive inoculations of a poly (I:C) influenza vaccine induce virus-specific CD8+ T cells in the lung.

Moriyama M, Takeyama H, Hasegawa H and Ichinohe T

The cytotoxic T lymphocyte (CTL) response plays a key role in host recovery from influenza virus infection and in subsequent immunity. Compared to natural infection with influenza virus, however, intranasal vaccination with adjuvant-combined inactivated vaccine elicits only moderate CTL responses. The reasons for this moderate effect are not fully understood. Here we demonstrate that 5 days of consecutive, intranasal vaccination with a combination of inactivated influenza vaccine and poly (I:C) elicits a strong CTL response in the lung. Although poly (I:C) did not stimulate uptake of antigens by respiratory dendritic cells (DCs), antigen-captured respiratory DCs did efficiently migrate from the

lung to the mediastinal lymph node (mLN) after the 5 day series of inoculations with vaccine and poly (I:C). Importantly, the intranasal poly (I:C) adjuvant-combined vaccination series stimulated proliferation of $CD8^+$ T cells in the mLN and recruitment of antigen-specific $CD8^+$ T cells into the lung. Although the CTL response was less effective against heterologous influenza virus, we show for the first time that intranasal administration of inactivated influenza virus vaccine and poly (I:C) for 5 consecutive days can elicit high levels of influenza virus-specific $CD8^+$ T cells in the lung.

3. The activation of NLRP3 inflammasome induced by SARS-CoV

Chen IY, Moriyama M and Ichinohe T

Severe acute respiratory syndrome associated coronavirus (SARS-CoV), the causative agent of SARS, can induce various cytokines release in the lung tissue of SARS's patients and in SARS-CoV-infected culture cells. The structural protein envelope (E) and the accessory protein 3a of SARS-CoV polymerize to form ion channels with its specificity of ionic conductance, such as sodium and potassium ion, and these viroporin properties have been suggested to be important for virus multiplication cycle. Viroporins, such as influenza virus M2 protein and EMCV 2B protein, can induce the activation of the NLR family, pyrin domain-containing 3 (NLRP3) inflammasome to induce the maturation of pro-caspase-1 and the release of mature interleukin IL-1beta (IL-1β and IL-18 in virus infection. Here, we demonstrated that the expression of E and 3a proteins stimulated secretion of IL-1β and the E protein partially colocalized with NLRP3. The ion channel activity-loss mutant of E protein, EV25F, and 3a protein, 3aCS, reduced IL-1β release induced by E and 3a protein, respectively. These results suggested the E and 3a viroporins are important in SARS-CoV-induced inflammasome activation.

4. Consecutive inoculations of influenza virus vaccine and poly (I:C) protects mice against homologous and heterologous virus challenge

Moriyama M, Chino S and Ichinohe T

Mucosal immunity induced through natural infection by influenza virus has potent cross-protective activity, compared to subcutaneous vaccination-induced systemic immunity. Compared to natural infection with influenza virus, however, a single intranasal vaccination with an inactivated influenza virus vaccine and poly (I:C) is not sufficient to induce primary immune response in naïve animals. The reasons for this moderate effect are not fully understood. Here, we demonstrated that intranasal vaccination with formalin-inactivated influenza virus vaccine and poly (I:C) for five consecutive days elicits high levels of virus-specific nasal IgA and serum IgG responses, while vaccination without poly (I:C) induced little response. Mice immunized with influenza virus vaccine and poly (I:C) for five consecutive days sustained high levels of virus-specific IgA in nasal wash and IgG in serum until at least 6 months after vaccination. Furthermore, intranasal vaccination with influenza virus vaccine and poly (I:C) protected mice against homologous and heterologous influenza virus challenge. These results suggest that consecutive inoculations of influenza virus vaccine and poly (I:C) is an alternative method to induce primary immune responses in naïve subjects.

Publications

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International Research Center for Infectious Diseases

Department of Infectious Disease Control Division of Bacteriology

感染制御系　細菌学分野

Associate Professor	Hitomi Mimuro, Ph.D.		准教授	医学博士	三　室　仁　美
Project Assistant Professor	Takahito Sanada, Ph.D.		特任助教	医学博士	眞　田　貴　人
Project Assistant Professor	Kotaro Kiga, Ph.D.		特任助教	医学博士	氣　駕　恒太朗

Bacteria-gut interplay and the host immune response are the most critical issues in determining the fate of bacterial infection and severity of the diseases. Our group has been studying pathogenesis of mucosal infectious bacteria, such as Helicobacter pylori, Shigella, enteropathogenic E. coli, and Streptococcus pyogenes, by defining the molecular and cellular mechanisms of infection and the roles of factors of pathogens and host in infection. The expected output of our research will not only shed further light into understanding bacterial pathogenesis, but also provide new paradigm in microbiology, cell biology, immunity, and pathology, and strengthen the molecular basis in developing diagnostic products, vaccines, animal models, and therapeutic agents.

1. *Helicobacter pylori* regulates BabA adhesion function by modulating Lewis b expression on the bacterial surface

Eisuke Kuroda, Nozomi Ishijima[1], Kotaro Kiga, Sayaka Hirukawa, Takahito Sanada, Zhenzi Piao[1], Reiner Haas[2], Chihiro Sasakawa[1,3,4], Tomas Bóren[5], and Hitomi Mimuro: [1]Department of Microbiology and Immunology, Institute of Medical Science, The University of Tokyo, [2]Max von Pettenkofer-Institut für Hygiene und Medizinische Mikrobiologie, Department Bakteriologie, Ludwig-Maximilians-Universität, Germany, [3]Nippon Institute for Biological Science, [4]Medical Mycology Research Center, Chiba University, [5]Medical Biochemistry and Biophysics, Umeå universitet, Sweden.

The persistent colonization of *Helicobacter pylori* (Hp) in the gastric pits of the stomach as well as the translocation of the bacterial cytotoxin-associated gene A (CagA) effector protein via the *cag*-Type IV secretion system (TFSS) into host epithelial cells are major risk factors for gastritis, gastric ulcers, and cancer. The blood group antigen-binding adhesin BabA mediates the adherence of Hp to Lewis b (Le^b) blood group antigens in the human stomach and triggers TFSS-dependent host cell signaling to induce the transcription of genes that enhance inflammation and intestinal metaplasia. Modification of BabA expression during Hp infection is thought to be a mechanism to adapt to changing conditions of inflammation and glycan expression at the epithelial surface. However, how Hp modulates BabA binding activity to the host for the long-term adaptation remains unclear. Here, we show *in vivo* that Hp binding activity to the host cell surface Le^b is diminished during long-term colonization of stomach. Challenge of Mongolian gerbils with Hp for 8 weeks yielded output strains that lost BabA-binding ability to Le^b, but expressed complete BabA protein. We found that the output strains expressed increased levels of Le^b on the bac-

terial surface polysaccharides. Breakdown of the bacterial surface Le^b structures by periodate oxidation or fucosidase treatment resulted in increased levels of bacterial binding to Le^b. These results indicate that Hp in the stomach modulates Le^b expression levels of its own surface to mask BabA adhesin to regulate adhesin function during infection.

2. Host E3 ubiquitin ligase limits pathogenicity of Enteropathogenic *Enteropathogenic E. coli*

Jin-Hyeob Ryu, Ryota Otsubo, Tamao Iida, Hiroshi Ashida[1], Chihiro Sasakawa[1,2], and Hitomi Mimuro: [1]Medical Mycology Research Center, Chiba University, [2]Nippon Institute for Biological Science.

Effector proteins are important regulators to hijack host cell process upon bacterial infection. Since the Tir-Intimin interaction-mediated adherence of EPEC to the infected epithelial cell is a key event for colonization as well as pathogenesis, we hypothesized that the host might possess suppression system specialized to protect from pathogen intrusion. In this context, we sought to evaluate host ability to eliminate Tir protein in infected epithelial cells. We demonstrated that the intracellular translocated Tir effector was rapidly degraded compared to other effectors via proteasome degradation pathway. We found that 454 tyrosine-phosphorylated Tir protein bound to host E3 ubiquitin-protein ligase, and the E3 ligase ubiquitinate Tir that lead to its proteasome-dependent degradation and attenuated EPEC colonization. We identified the E3 ubiquitin-protein ligase as a host maneuver to degrade EPEC-infected Tir effector to eliminate their colonization.

3. A small RNA regulates oxidative stress response by stabilizing Trx2 mRNA in *Helicobacter pylori*.

Kotaro Kiga, Bo Zhu, and Hitomi Mimuro

Helicobacter pylori (*H. pylori*) is a spiral-shaped, microaerophilic bacterium that is mainly found in the stomach. During the infection process, *H. pylori* is always exposed to oxidative stress from oxygen in the atmosphere or reactive oxygen produced from host immune cells. Although *H. pylori* might have developed several survival systems to protect themselves from oxidative stress, the mechanisms still remain unclear. In this study, we identified a bacterial small RNA (sRNA) important for *H. pylori* cell survival under oxidative stress. Knockout mutant of RepG sRNA was more susceptible to oxidative stress than wild-type. We analyzed gene expression pattern by RNA-Seq and figured out that RNA expression level of Thioredoxin-2 (*Trx2*), which is known as an important factor for cell survival under oxidative stress, was decreased in the RepG-deletion mutant compared with wild-type. Further experiments identified the RepG directly binds to the 5'-end of *Trx2* mRNA to prevent the mRNA degradation. Indeed, the sRNA and *Trx2*-dependent *H. pylori* survival was confirmed in macrophages that produce reactive oxygen species. Taken together, our results suggest that the sRNA plays an important role in the *H. pylori* survival under oxidative stress, which might be important for bacterial immune evasion.

International Research Center for Infectious Diseases

Pathogenic Microbes Repository Unit
病原微生物資源室

Associate Professor Hitomi Mimuro, Ph.D.

准教授 医学博士 三 室 仁 美

This unit is collecting standardized bacterial strains and distributing to research organizations, hospital laboratories, and medical educational institutions throughout the country. In addition, under cooperation with the Japanese Society for Bacteriology, we are distributing authorized bacterial strains for microbiology course for medical school.

Our society is always threatened by emerging and reemerging infectious diseases with various kinds of altitude pathogenic microbes owing to increased foreign tourism, import increase including food, food poisoning such as the O-157 epidemic, and bioterrorism. In addition, by advanced medical developments, the aging society, and increased HIV infection, the quick identification of and therapy for opportunistic infection causative agents and multiple drug resistance bacteria have become important in the medical field.

The need for researchers and clinical practitioners specialized in bacteriology and infectious diseases have risen remarkably, and the substantial study and education required is an emergent problem. For thorough study and education, knowledge of bacteriology, a system of collecting pathogenic microorganism strains of reliable origin, to maintain and save them appropriately, and to provide them to cutting-edge researchers or educational establishments is indispensable. However, in Japan, research into pathogenic microorganisms and infectious diseases is performed mainly in universities, where there is no system for conservation and supply. Therefore, valuable bacterial strains have faced disappearance. Furthermore, under the CARTAGENA PROTOCOL ON BIOSAFETY for conventions of biological diversity, the provision and purchase of pathogenic microorganisms from foreign countries has become difficult.

In such circumstances, we are collecting, saving, and analyzing the pathogenicity of microorganisms and distributing pathogenic bacteria to 1) offer type cultures as a positive control in research, education and examinations, 2) prepare pathogenic bacterial strains that have socially high importance, and 3) offer microbes to universities or public research organizations for training or research. We possess about 1,500 strains that almost cover the main pathogenic microbes, including strains valuable internationally such as pathogenic *E. coli* of Orskov's collection, which is stored only in our laboratory in Japan. Furthermore, it is important to secure their utility as type cultures by preparing genomic and genetic information about the pathogenicity of our bacterial collection based on the researches of the Division of Bacterial Infection. Thus, our laboratory is expected to contribute to countermeasures against infectious disease, and to the education and research of medical microbiology in our country.

Collection, preservation and data management of bacterial strains

It is necessary for us to collect representative type strains and the derivatives of pathogenic microbes corresponding to the following six items.

a) Comprehensive collection of genome sequencing

strains.

b) The causative agents of hospital-acquired (nosocomial) infection, such as opportunistic infectious bacteria and antibiotic-resistant bacteria.
c) Pathogenic *Escherichia coli* associated with the intestinal and urinary tract or meningeal infections, including *Shigella*, EPEC and EHEC O-157.
d) Intracellular bacterial pathogens such as *Mycobacterium avium* and obligate intracellular bacteria.
e) Zoonotic agents causing brucellosis (*Brucella*), leptospirosis (*Leptospira*), and so on.
f) Pathogens causing newly emerging infections and outbreaks, such as *Helicobacter pylori*, *Salmonella* spp. and *Clostridium* perfringens.

We dissect the biochemical properties of bacterial strains collected by deposition, and maintain them appropriately. We are also opening the database of our collection to the public.

Distribution of bacterial strains

We are distributing standardized bacterial strains to research organizations, hospital laboratories, and medical educational institutions throughout the country. In addition, under cooperation with the Japanese Society for Bacteriology, we are distributing authorized bacterial strains for microbiology course for medical school.

Value-added creation of a bacterial strain collection by pathogenic analysis

We are analyzing the pathogenicity of pathogenic microorganisms, especially pathogenic *E. coli*, the pathogenicity of new bacterial infection causative agents in cooperation with the Division of Bacterial Infection. Our collection has original added value by offering this information to users.