Epidemiological study on *Staphylococcus aureus* in a laboratory animal facility using genotyping

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*Staphylococcus aureus* strains isolated from mice, rats, and Syrian hamsters at the National Defense Medical College Experimental Animal Facility were genotyped by multilocus sequence typing (MLST) and *S. aureus* protein A gene typing (*spa*). Four genotypes were confirmed in mice, 3 in rats, and 3 in Syrian hamsters in MLST. Eight genotypes were confirmed in mice, 3 in rats, and 2 in Syrian hamsters in *spa*. There was no genotypic overlap between species, despite the ability of interaction in the laboratory. Most of the genotypes were segregated throughout the study. In rat and Syrian hamster-derived strains, genotypes varied in respective animal species by only one or two bases in the target MLST genes. One mouse-derived genotype strain was a methicillin-resistant *S. aureus* (MRSA). This strain appeared in a mouse room in 2018 and then spread throughout the facility’s mouse rooms. This MLST type includes community-acquired MRSA and livestock-associated MRSA. These results suggest that the isolated *S. aureus* strains have high specificity for their host of origin and have been established in the facility for a long time. However, as community-acquired and livestock-associated MRSA are now frequently isolated, the chances of bringing MRSA with an affinity for experimental animals into experimental animal facilities are expected to increase. Therefore, the significance of monitoring *S. aureus* is becoming increasingly important.
ICLAS Performance Evaluation Program for accuracy control of microbiological monitoring testing

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In laboratories that conduct microbiological monitoring of laboratory animals, it is important to control the accuracy of testing. In these laboratories, it is possible to perform accuracy control using a standard panel of samples produced in-house by the laboratory. However, there is a problem in that there are few opportunities to confirm the accuracy of testing using samples derived from the field. In 2007, the International Council for Laboratory Animal Science (ICLAS) provided the Performance Evaluation Program (PEP), a self-evaluation program for microbiology laboratories, to solve this problem. This program blinds bacterial strains, sera, and microorganism-derived nucleic acids prepared in five network laboratories designated by ICLAS and distributes them to program participants. In this program, participants can test these samples using in-house methods and protocols and request the expected results from the PEP Secretariat when a definitive diagnosis has been reached. In addition, participants can get advice as needed from the above five network laboratories. PEP is able to perform quality control using microbial strains and derived samples isolated in various places around the world. It is considered useful for control of accuracy in microbiological monitoring.
Examination to reduce non-specific reaction in changing chromogenic substrate solution of MONILISA®

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MONILISA® is the kit for microbiological monitoring tests of experimental animals. In 2018, we compared 3,3',5,5'-tetramethylbenzidine (TMB), a less toxic reagent to OPD that is used for the kits currently, and reported use of TMB causes non-specific reactions. In this study, we examined condition of concentration and volume of antigen and concentration of masking reagent to reduce non-specific reaction when used TMB as a chromogenic substance. Coated antigen conditions are following 3 types, current condition, condition1 (current 1/2 concentration, current 1/2 volume), condition 2 (current concentration, current 1/2 volume). Skim milk (0.1% and 5%) was used for a masking reagent. Following five items were tested for each six mice and rats serum sample that showed non-specific reaction by MONILISA®; Sendai virus (HVJ), Mouse Hepatitis virus (MHV), Mycoplasma pulmonis (Myco), Clostridium piliforme (Tyzzer) and Hanta virus (HANTA). As the results, using 5% skim milk showed reduction of non-specific reactions. The sample numbers in which non-specific reactions disappeared is as follows, Condition 1 ; HVJ (rats 3/6), MHV (mice 5/6) (rats 6/6), Myco (mice 2/6) (rats 4/6). Condition 2 ; Tyzzer (mice 4/6) (rats 1/6), HANTA (rats 6/6). From these results, using 5% of skim milk and optimal concentration and amount of antigen is useful to reduce non-specific reactions when use TMB as chromogenic reagents in MONILISA®.
Spontaneous left torticollis with weight loss was detected in a 9-week-old, male DBA/2NCrl mouse that had been used in the background data analyses in young DBA/2NCrl mice. Histologic evaluation revealed exudative inflammation around the pharyngeal orifice of eustachian tubes, and purulent inflammation of both middle ears and left inner ear with abscess formation and clusters as gram-positive microcolonies. *Staphylococcus aureus* (*S. aureus*) was identified from swab cultures of both external ear canals. As a result, this case of torticollis in a DBA/2NCrl mouse was caused by *S. aureus*-suppurative otitis media and interna. *S. aureus* is known to an opportunistic pathogen with the potential to frequently cause suppurative diseases to immunocompetent laboratory animals. However, to our knowledge, this case presented with torticollis in a DBA/2NCrl mouse is previously unreported *S. aureus* infectious disease states.
Transcriptome analysis of trichomonads parasitizing in mouse cecum

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Murine trichomonads that are distinguishable by rRNA sequence (ITS1/5.8S rRNA/ITS2) are \textit{Tritrichomonas muris}, \textit{T. musculis} (2015) and \textit{T. rainier} (2016). We reported the 4th murine trichomonad (\textit{Tritrichomonas} spp. X260) at the 66th JALAS meeting, 2019. Here we describe our comprehensive transcriptome results obtained from RNA-seq of murine cecum parasitizing trichomonad (\textit{Tritrichomonas} spp. AC624) in order to find out common and/or specific gene sequences among these trichomonads.

[Materials and methods] Trichomonads were separated by Percoll plus centrifugation and RNA was extracted by using RNAs-icil-P (Rizo) and RNeasy column (Qiagen). TruSeq Stranded mRNA Kit (Illumina) was used to prepare the sequencing library and around 60 million clusters were read. [Results and discussion] An initial de novo transcriptome assembly was generated by Trinity. More than 70\% of genes were found in eukaryotic conserved core gene set by BUSCO analysis and homology search revealed that 81.4\% of the predicted genes were found in related \textit{T. foetus} genes. Sequence of rRNA operon was highly identical to the sequence of PCR products from genomic DNA. Four house keeping genes (ex. GAPDH) were found in the transcriptome contigs. It is said that almost of protozoan genes lack introns. The sequence data obtained from this study will be useful to design PCR primers against common and/or specific genes of murine trichomonads. This work was supported by the NBRP Fundamental Technologies Upgrading Program.
Comparison of colonization dynamics of altered Schaedler flora in three mouse strains

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The altered Schaedler flora (ASF), consisting of eight bacterial species, is common globally as minimum microbiota that is sufficient to establish normal physiological functions in the gut of mice. Recently, we introduced ASF, and started the basic study for global harmonization. In this study, we investigated the colonization and distribution dynamics of 8 strains in the gut of gnotobiotic ASF-colonized C57BL/6J, C57BL/6N and BALB/cA mice.

The colonization change of the ASF strains caused by aging was assessed using fecal samples obtained each week from 6 male and 6 female 3-12-week-old each strain mice. Stomach, small intestine, cecum, colon samples were collected from same mice at 12-week-old to analyze the distribution patterns of the ASF strains. Extracted DNA from each sample was used for real-time quantitative PCR based on SYBR Green I.

As a result, 3-week-old mice showed variable bacterial counts in several strains of ASF. Except for ASF 360, the numbers of bacteria of all ASF strains were no rapid change in each mouse strain after 4 weeks of age. In addition, we will evaluate distribution changes of the ASF strains in gut of each mouse strain. Then, we will present the data including differences in composition of ASF strains due to mouse strain.
Development of new antibacterial monoclonal antibodies against Pseudomonas aeruginosa

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Introduction

Immunodeficient experimental animals have been extensively developed today, but the risk of death is high when they are affected by opportunistic infection. Today, multidrug-resistant \textit{P. aeruginosa} is increasing and the precaution and medical care of experimental animals are demanded. We thought it effective to apply antibody therapy and focused on BAM complex, which is located in adventitia of \textit{P. aeruginosa}, one of the main and vital components found in Gram-negative bacteria. As BAL6 has a projecting loop outside of BAM complex, we prepared monoclonal antibodies against it.

Materials and Methods

BALB/c mice were immunized with BAL6MAP emulsified with Freund’s adjuvant, and hybridomas were prepared by conventional PEG method. Specific hybridomas were screened by ELISA and the specificity was confirmed by measuring cross-reactivity to a 3rd party antigen, named CH401MAP. The binding avidity to intact bacteria antigen was measured by binding assay.

Results and Discussion

We obtained two clones of BAL6MAP specific mAb, which react with intact \textit{P. aeruginosa} but not with CH401MAP. The subclass of these mAbs was IgG1 (κ). We are now analyzing whether these mAbs can induce innate immune system to prevent the death of the immunodeficient mice. These mAbs are promising for the analysis of localization and the function of BAM complex.
Inhibition effects of human leukocytes induced by Glucocorticoid using NOG-hIL-4-Tg

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Glucocorticoid (GC) is a steroid hormone commonly used as an immnosuppressant. GC suppresses T cell activation by reducing IL-2 production. While B cells are expected to decrease antibody production in the presence of GC by the T cell malfunction, no available system of long-term culture of B cells to analyze the effect of GC to B cell function has been explored extensively. Therefore, we tried to establish the \textit{in vivo} system using NOG-hIL-4-Tg for the analysis. Human PBMCs were cultured in the presence of cortisol for 6 hours and transplanted into NOG-hIL-4-Tg mice. The mice were immunized with CH401MAP every 2 weeks and analyzed by flow cytometry (FCM) 4 weeks after the transplantation. As an in vitro control, hPBMCs were stimulated by TSST-1 in the presence of cortisol for 3 days and the cellularity was analyzed by FCM. The expression of T cell activation markers was decreased in a dose dependent manner \textit{in vitro}, while plasmablasts were tended to decrease. The expression of T cell activation markers and plasmablast number were tended to decrease in the NOG-hIL-4-Tg spleens. These results suggest that NOG-hIL-4-Tg mouse might mimic the effect of GC observed \textit{in vitro}, while it maintains lymphocyes to enable long-term observation.
Dietary fiber is fermented to short-chain fatty acids (SCFAs) by microbiota in the colon. SCFAs are known to serve as a fuel source of colonic epithelia and affect adipocyte size and function. In addition, recently, SCFAs also influence the immune homeostasis. GPR43 (known as Ffar2) is one of the major receptors of SCFAs and expressed on adipose, intestinal, and immune tissue. However, the function of GPR43 in T cell responses remains unclear. In this study, we examined the role of GPR43 in T cell responses using GPR43 deficient (GPR43KO) mice. In acute Graft versus Host Reaction (aGVHR), donor T cells from GPR43KO mice developed severe symptoms. We also found that frequency of IL-2 producing T cells from GPR43KO mice was higher than those from WT mice in vitro experiments. Bone-marrow chimera experiment revealed that the expression of GPR43 on non-hematopoietic cells but not on T cells was responsible for regulating cytokine production of T cells. Furthermore, feeding of high fiber diet decreased the population of IL-2 producing T cells. These results suggest that GPR43 sufficient environment in vivo may instruct T cells to limit excessive T cell responses.
Induction of specific immunoglobulin in middle ear mucosa by intranasal immunization.

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The interactions between nasopharyngeal mucosae and adjacent tubotympanal or tracheal mucosae are focused on in otolaryngeal field. In order to clarify these interactions, we were successful to provoke mucosal immune responses (immunoglobulin A (IgA) and immunoglobulin G (IgG) response) in nasopharyngeal mucosae by repeated intranasal chicken ovalbumin (OVA) challenges with cholera toxin (CT) in wild type BALB/c mice and also even without CT in TCR-transgenic mice (OVA-23-3). We have also observed the OVA-specific mucosal immune response in tubotympanum against middle ear antigenic stimuli. These results indicate that OVA specific IgA immune response was induced in middle ear mucosa by intranasal immunization. Our data in OVA-23-2 mice without adjuvant confirmed that the interaction between nasoparyngeal mucosae and tubotympanal mucosae as regard mucosal immune response is actually present in a murine system. And our data that OVA specific IgA immune response could be induced in the middle ear mucosae in OVA-23-2 mouse without adjuvant suggest that tubotympanal mucosae is one of the member of commom mucosal immune system (CMIS) and there is a close interaction with nasopharyngeal mucosae.
The role of IL-36alpha in the development of imiquimod-induced psoriasis and DSS-induced colitis

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IL-36alpha, an inflammatory cytokine (gene symbol Il1f6), is involved in the activation of dendritic cells, the differentiation of T cells and the proliferation of keratinocytes (KCs) by transducing signals through formation of the hetero-complex of IL-36 receptor (IL-36R) and IL-1 accessory protein. Emerging evidences have suggested that Il1f6 is closely associated with inflammatory diseases such as psoriasis and colitis. However, the role of IL-36alpha in the development of these diseases has been poorly understood. In this study, we found that IL-36alpha-deficient mice developed milder psoriasiform dermatitis than WT mice upon treatment with imiquimod-cream, whereas the susceptibility of dextran sodium sulfate-induced colitis was similar between WT and IL-36alpha-deficient mice. Bone marrow transfer revealed that skin IL-36alpha plays important roles for the pathogenesis of psoriasiform dermatitis. We found that Il1f6 was produced in KCs, whereas IL-36R gene was expressed in KCs, bone marrow-derived Langerhans cells (BMLCs) and fibroblasts. Upon stimulation with IL-36alpha, Il1f6 was induced in KCs and BMLCs, but not fibroblasts. Also, IL-36alpha stimulation induced psoriasis-associated genes in KCs, BMLCs and fibroblasts. These suggest that skin IL-36alpha is involved in the development of imiquimod-induced psoriasiform dermatitis by forming local inflammatory amplification.
HIV infection in NOG-Alb KO mice and PK analysis of anti-HIV compounds

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Protein binding is an important factor which affects pharmacokinetics/pharmacodynamics (PK/PD) of drugs. We have previously reported establishment of albumin deficient NOG (NOG-Alb KO) mice (Suemizu H. et al, 2018). Characterization of NOG-Alb KO mice was performed since human immunodeficiency virus (HIV) infection and PK/PD analysis of anti-HIV drugs using this strain could contribute to predict efficiency in human. We confirmed that three groups of NOG-Alb KO mice were successfully engrafted with human hematopoietic stem cells. HIV (NL432 strain) infection was established in humanized NOG-Alb KO mice. Since some mice showed slow viral growth and which was not observed in normal humanized NOG mice, further analysis will be needed to determine the cause. Anti-HIV drugs (raltegravir and compound A) were administered to NOG and NOG-Alb KO mice, and clear difference in PK was observed. Since albumin in NOG mice was considered to contribute to sustained plasma concentration of tested compounds, NOG-Alb KO mice with additionally expressing human albumin could be more useful model for PK/PD analysis of anti-HIV drugs.
The aim of this study is to demonstrate the efficacy of hepatitis E virus-like particles (HEV-LP) as a vaccine in rat HEV infection model using rats.

METHODS: Rat HEV capsid protein was expressed in E. coli and purified to prepare rat HEV-LP. Rat HEV-LP was intramuscularly inoculated with TiterMax Gold or Alhydrogel adjuvant into F344 rats at 20-80 μg/shot 3 times every 2 weeks. The vaccinated rats were orally infected with 10^6 or 10^7 copies/rat of rat HEV, followed by measuring rat HEV RNA in feces and sera and serum antibodies.

RESULTS: In the immunized rats, high titer of anti-rat HEV ORF2 IgG was induced regardless of the type of adjuvant. When the vaccinated rats were orally infected with 10^7 copies/rat of rat HEV, 100% infection prevention effect was shown even in the 20 μg/shot vaccination group.

CONCLUSION: Rat HEV-LP demonstrated strong effect as vaccine in rat HEV infection model. These results may promise future development of HEV-LP vaccine for HEV infections in pigs and humans.
A survey for Rat polyomavirus 2 in laboratory rats in Japan

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Recently, Rat polyomavirus 2 (RatPyV2) has been identified in a colony of immunodeficient (XSCID) rats. In this study, we revealed detailed pathology of RatPyV2 in XSCID rats, surveyed RatPyV2 infection in immunocompetent laboratory rats in Japan, and evaluated the utility of the LAMP method for detecting RatPyV2 gene. Histologically, we observed basophilic intranuclear inclusion bodies within epithelial cells in the salivary, Harderian, and extraorbital lacrimal glands, and in respiratory and reproductive tissues. The parotid salivary glands were the most severely and diffusely affected. We detected RatPyV2 genes in DNA samples including buccal swabs or blood smeared on the FTA cards. In serological test, 43 in 111 immunocompetent rats (38.7%) were positive for RatPyV2. PCR test by the Amp-FTA method revealed that 43.8% immunocompetent rats were positive for RatPyV2 genes. No pathological changes were detected in these rats. The LAMP method using buccal swabs detected RatPyV2 gene in XSCID rats. It was suggested that the salivary glands were the most highly susceptible tissue to RatPyV2. Our survey for RatPyV2 revealed relatively widespread RatPyV2 infection in immunocompetent laboratory rat populations in Japan. Finally, the Amp-FTA and LAMP method using buccal swabs could be useful for the rapid diagnosis of RatPyV2.
Development of an ELISA for rat polyomavirus 2 antibody and test the rat blood for antibodies

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Severely immunodeficient X-SCID rats were bred to establish a colony. During breeding, the rats exhibited dyspnea and died by infection of a novel rat polyomavirus 2 (RatPyV2). There were concerns about spread of infection to other rodents, hence SD rats, nude rats and NOG mice were examined. Infection was detected in immunodeficient rodents, but not in SD rats, implying immune function may protect from infection. So an ELISA for RatPyV2 antibody was developed to test rat serum. For the antigen preparation, viral particles were purified from the infected tissues (salivary glands, lung, and uteri) by sucrose density-gradient centrifugation, and finally 1.53 mg as protein amount was obtained. ELISA construction kit for rat (Beacle, Inc, Kyoto) was obtained, and the optimal antigen concentration at 10 μg/mL and antibody concentration range from 100- to 30,000-fold dilution was determined. Serum samples collected from experimentally inoculated SD and nude rats, Wistar rats fostering X-SCID neonates, Wistar juveniles fostered by X-SCID rats and naive SD rats were tested by ELISA for RatPyV2 antibody. Antibody was detected in SD, Wistar foster mothers and juveniles, but not in naive SD or nude rats. These results suggest that this ELISA is useful to detect RatPyV2 antibody, and that antibody is raised and protects against RatPyV2 infection in immunocompetent rats.
Attempt to generate hairless severe immunodeficient NOD/Scid/Jak3null Mouse

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NOD/Scid/Jak3null (NOJ) mouse is a highly immunodeficient mouse strain with complete loss of T, B, NK cells. They are expected to be applied to research on human diseases such as human specific infections and cancer. While, hairless mice have normal immune system but they have the advantage of easy to observe tumors subcutaneous transplantation. In this study, we attempt to generate hairless NOJ from NOJ mice and hairless mice, and we assessed blood cells composition or transplantation rate of these mice. Male hairless mice were used as sperm donors. Female NOJ mice were used as oocyte donors and they were induced superovulation. We performed an in vitro fertilization and embryo transfer. Tail samples from babies were extracted genomic DNA and performed genotyping. We performed the above procedures and repeated until we gain hairless homozygous. Spleen from hairless NOJ were stained by immune antibody and analyzed by flow cytometry. Hairless NOJ were subcutaneously inoculated with malignant lymphoma and human leukemia cell lines. It wasn't observed T, B, NK cells in hairless NOJ and they developed subcutaneous tumors. These results may indicate that hairless NOJ have immune system same as NOJ mice.
We reported whole genome sequence of Rodentibacter heylii (ATCC:12555\textsuperscript{T}) and R. pneumotropicus (JCM:14074\textsuperscript{T}) at the JALAS meeting 2019. We observed JCM:14074\textsuperscript{T} had a circular bacteriophage DNA and the both strains possessed many phage-related genes in their genomes. Since then, we have performed resequencing of R. heylii and R. pneumotropicus isolates. Here we describe the correlation between bacteriophages and Rodentibacter species. [Materials and methods] Strains isolated at RIKEN (3 of R. heylii and 4 of R. pneumotropicus) were used for resequencing. Already deposited genome sequences were downloaded from NCBI. [Results and discussion] Circular phage DNA of JCM:14074\textsuperscript{T} was typed as Haemophilus virus HP1 by virus detecting site (https://www.genomedetective.com). In the same way, four prophages were predicted in ATCC:12555\textsuperscript{T} genome and their virus typing was performed. Paying attention to phage-related sequences, we further analyzed the resequencing data and found that the number and type of prophage in those genomes differed among the species and the strains. Furthermore, short parts of phage DNA sequences existed in the spacer regions of their CRISPR array. These results suggest the phage-related DNA sequence can be used as a genetic marker to identify the individual strain. Using these results, we will develop simple methods to distinguish the isolates in a strain-specific manner. This work was supported by the NBRP Fundamental Technologies Upgrading Program.
Characterization of \textit{Rodentibacter} sp. isolated from rodents.

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Currently, \textit{Pasteurella} pneumotropica has been reclassified as new genus \textit{Rodentibacter}. Of these, \textit{R. pneumotropicus} and \textit{R. heylii} are main target for microbiological monitoring in rodent. We have isolated a number of \textit{Rodentibacter} sp. that could not be identified as \textit{R. pneumotropicus} and \textit{R. heylii} from rodents. Hereafter, these isolates might be continuously isolated in microbiological monitoring. In this study, we explore characteristics of \textit{Rodentibacter} sp. isolate, with focusing on bacterial virulence. \textit{Rodentibacter} sp. that isolated from rat trachea were used for the study. Draft genome sequencing was performed on next generation sequencer, and then, the virulence-associated genes were predicted. In animal experiments, Rag2 and CD1dKO mice were used for \textit{Rodentibacter} sp. infection. By the draft genome sequencing, unique RTX toxin-coding gene had been identified in \textit{Rodentibacter} sp. Further, animal experiments revealed that one of 5 Rag2 mice was dead in 1 month after infection; otherwise, all the CD1dKO mice was survived. Although the virulence-associated genes were identified, certain pathogenicity could not be determined in animal experiments. Furthermore, virulent characterization toward immunodeficiency mice might be heterogeneity.
Human T-cell leukemia virus type 1 (HTLV-1) causes relevant diseases including adult T-cell leukemia in a part of carriers. However, anti-HTLV-1 therapies in carriers are not developed. We previously confirmed usefulness of our HTLV-1-targeting virotherapy with a novel drug candidate, a recombinant vesicular stomatitis virus (rVSV), in a humanized mouse model for HTLV-1 infection. This rVSV expressing HTLV-1 receptor targets and eliminates HTLV-1-infected envelope proteins (Env)-expressing cells via Env-receptor interaction. Thus, in this study we aimed to examine whether Japanese macaques naturally infected with simian T-cell leukemia virus type 1 (STLV-1) close to HTLV-1 can be applied to a primate model to evaluate the rVSV. Based on our recent sequence analysis of STLV-1 whole genomes, we constructed Env expression plasmids for 4 STLV-1 strains, transfected Vero and 293T cells with these plasmids, and checked cell fusion activity of STLV-1 Envs. Among Env-expressing cells, we found the highest activity of Si-2 Env in Vero cells. Our present findings indicate that STLV-1 infects susceptible cells in the same way as HTLV-1 and that STLV-1-infected Japanese macaques can be applied to a primate model to develop our rVSV-based HTLV-1-targeting virotherapy.
Evaluation of vertical infection of pregnant marmosets with Zika virus

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[Introduction] Zika virus (ZIKV) causes fetal abnormality, when pregnant women are infected with ZIKV. To establish ZIKV vertical infectious model, we evaluated the vertical transmission of pregnant marmoset with ZIKV to the fetus.

[Methods] Three pregnant marmosets at gestational day 50-60 (G50) and one pregnant marmoset at gestational day 95-110 (G95) were inoculated with ZIKV.

[Results] Spontaneous abortion appeared in two G50 marmosets. The G50 marmosets except for the 2 d.p.i.-abortion marmoset and the G95 marmoset were euthanized at 8 d.p.i. and 9 d.p.i. respectively. Viral RNA copies were detected in organs of fetus, amniotic fluids, placentas, umbilical cords and maternal uterus. Viral antigens were detected in maternal endometrial cells of G50 marmoset.

[Discussions] The results suggested that ZIKV propagated in endometrial cells and induced abortions in G50 marmosets. Furthermore, the results suggested that fetuses would be infected with ZIKV, when marmosets were infected with ZIKV during pregnancy. This pregnant marmoset model allows us to study the mechanism of mother-to-fetus ZIKV infection.