ADC Letter
for Infectious Disease Control

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TEIKYO

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## EDITORIAL

On the occasion of publication of Volume 3 No.1
Kazuo Suzuki, Director
Asia International Institute of Infectious Disease Control, Teikyo University (ADC)

1. 本号からPeer Review付きのOriginal Articleを掲載することになりました。
We start a new contents peer-reviewed Original Article from this issue.
2. アジア諸国との医療・研究の連携をめざして、感染症研究・医療の研修会を行い好評のうちに終わりました。
2016年度は、帝京大学医学部学生が感染症研究・医療の研修会にアジア諸国を訪問します。第1回は、ベトナムの国立病院を訪問する予定です。

This issue contains topics in training course for communications between Asia countries and ADC Institute supported by SAKURA Science Plan of JST. This year ADC will organize training for Teikyo University Medical Students in Asia country Vietnam for one week supported by Teikyo University Scholarship (TAVP Scholarship).

at e-ASIA Project Meeting in Bangkok, Oct. 10 2015

このように、Asia International Institute of Infectious Disease Control, Teikyo Universityでは、「アジア諸国」と「感染症」をキーワードとして医療・研究の連携をめざしてゆきます。2015年ノーベル医学・生理学賞を受賞された北里大学・大村智先生や東京大学・北徹先生をはじめ、アジア・アフリカでの感染症への治療や施策にご尽力している先生方との共同研究や、大阪大学・木村英作先生との交流を通じてアジア・アフリカの医療状況を学んでいます。

ADC staff has collaborations with Prof. Satoshi Omura (Nobel Prize), Prof. Kiyoshi Kita in the University of Tokyo, Prof. Kimura in Osaka University, and other distinguish investigators.

また、ADC研では、京都大学iPS研究所・長船健二先生、北里研究所・砂塚敏明先生と赤川清子先生、東京都長寿医療センター・濱野慶朋先生、国際医療福祉大学・湯村和子先生、千葉大学・中山俊憲先生や石和田稔彦先生、北野病院・武曽恵理先生、京都大学・猪原登志子先生、聖マリアンナ医大・川上民裕先生、ルイ・パストゥール医学研・宇野賀津子先生と共同研究を進めています。本学では、TARC・寺本民生先生、小児科・三牧正和先生や、内田俊也先生、河野肇先生をはじめ、ADC教授会メンバーの先生方にご協力いただいております。

これらの成果を業績として残るように本巻からPeer Review付きのOriginal Articleとして発刊することにいたしました。

今後ともADC Letter for Infectious Disease Controlをご愛読、ご投稿のほどよろしくお願いいたします。

# 日本・アジア青少年サイエンス交流事業「さくらサイエンスプラン」【研修記録】

# Records of TASP Training Supported by SAKURA Science Plan of JST

2015年12月14日

帝京大学 アジア国際感染症制御研究所 所長 鈴木和男
Asia International Institute of Infectious Disease Control, Teikyo University, Director & Professor

## 研修参加者 Visitors for TASP Training Supported by SAKURA Science Plan of JST

| | | |
|---|---|---|
| ベトナム3名 | Nguyen Minh Hang | Researcher |
| | Nguyen Thi Ngoc Tran | Pediatrician |
| | Doan Thi Mai Thanh | Pediatrician |
| ラオス1名 | Arounnapha Vongdouangchanh | Researcher |
| フィリピン3名 | Inez Andrea P. Medado | Researcher |
| | Hannah Leah E. Morito | Researcher |
| | Neil Andrew Bascos | Assistant Professor |

2015年11月16日から12月4日の間、JST（科学技術振興機構）「さくらサイエンスプラン」ベトナム、フィリピン、ラオスの3ヵ国から7名の研修生を受け入れ、研修プログラムを実施しました。

本事業の目的に沿い、科学技術の分野でアジアと日本の青少年が交流を深めるため、大学院生、研究員・教員、医師を帝京大学アジア国際感染症制御研究所（ADC）に招聘しました。

今回は研究者として自国で活躍する7名が来学し、「感染症」と「安全管理」をテーマとした研修として来学しました。

We organized SAKURA Science Plan supported by JST from Nov. 16 to Dec. 4 in 2015 in ADC Teikyo University.

その内容をテーマ別に研修を行いました。Contents of the Training

1. 冲永佳史学長挨拶およびADC研教授会での紹介
   Introduction of the President Yoshihito Okinaga and Professors in ADC and ADC Staff
   鈴木和男（所長）、斧 康雄教授（医学部微生物学講座）、古川泰司教授（医学部附属病院中央検査部）、槇村浩一教授（医療共通教育研究センター）、高橋秀依教授（薬学部）、松永直久講師（医学部附属病院感染制御部）、井上まり子講師（公衆衛生学）、高橋和浩講師（医学部小児科）、ADC研スタッフ、医学研究科大学院生（ADC研、他）
2. 講習会：バイオセーフティ、医療安全
   Trainings and Lectures: Biosafety and Safety Control in Hospital
3. 実験室研修：ADC感染症研究室、斧研究室、槇村研究室
   Trainings in Laboratories
4. 薬学部研修
   Training in Faculty of Pharmacy
5. 講義：感染症、公衆衛生学
   Lectures: Infectious Diseases and Public Health
6. 特別講演会：リベリアでのエボラ対策支援、劇症型インフルエンザ
   Special Lectures: Ebola and Influenza
7. 医学部附属病院ラウンド：病院長、看護部、感染制御部、中央検査部、薬剤部、患者相談室の視察
   Tour of Teikyo University Hospital
8. 医学部4年生への来年度公衆衛生学実習ガイダンス：ベトナムでの感染症実習
   Joining with Medical Students in Teikyo University 4-year's guidance
9. 学外：ADC研と連携している結核研究所訪問
   Visiting RIT
10. 冲永佳史学長からの研修修了証授与および歓送会
    Certificate Celebration and Farewell Party

## 謝辞 Acknowledgements

冲永佳史学長、ADC教授会メンバー（斧 康雄、古川泰司、槇村浩一、高橋秀依、松永直久、井上まり子、高橋和浩の各先生方）、医学部、薬学部の教員のみなさま、病院スタッフ（藤森 新院長、坂本哲也副院長、高田眞二講師、渡邊真知子薬剤部長、土谷明子看護部長、内田れい子相談室長）、安全管理部、感染制御部、中央検査部、看護部、薬剤部および相談室のみなさま。

## 1．冲永佳史学長挨拶およびADC研教授会での紹介
Introduction of the President Yoshihito Okinaga and Professors in ADC and ADC Staff

**The President and ADC Executive Members**

The President Yoshihito Okinaga and Director Kazuo Suzuki

Dr. Takahashi and Visitors

Prof. Ono

Prof. Furukawa

Prof. Makimura

Dr. Matsunaga

Dr. Inoue

## 2．講習会　Trainings and Lecture

### 1）バイオセーフティ（Biosafety）

Biosafety Lecture by Prof. Suzuki

### 2）医療安全（Trainings: Safety Control in Hospital）


With Staff of Safety Control Department

## 3．実験室研修　Trainings in Laboratories

### 1）ADC感染症研究室（ADC Lab）

### 2）斧研究室（Prof. Ono's Lab）

### 3）槇村研究室（Prof. Makimura's Lab）


## 4．薬学部研修　Training in Faculty of Pharmacy


With Prof. Takahashi and Dr. Tabata

## 5．講義　Lectures

### 1) ADC

Biosafety Lecture by Dr. Ito

### 2) Public Health

Public Health Lecture by Dr. Inoue

## 6．特別講演会　Special Lectures

**Special Lectures**


## 7. 医学部附属病院ラウンド　Tour of Teikyo University Hospital

### 1）藤森新病院長と帝京大学医学部附属病院

With Director of Teikyo University Hospital
Prof. Shin Fujimori

Teikyo University Hospital

### 2）看護部（Nurses Units）

With Head of Nurse Ms. Tsuchiya

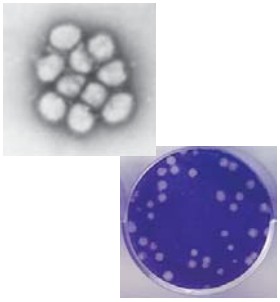

### 3）感染制御部（Infection Control Department）

With Staff of Infection Control Department

### 4）患者相談室（Mediation Section）

With Ms. Uchida

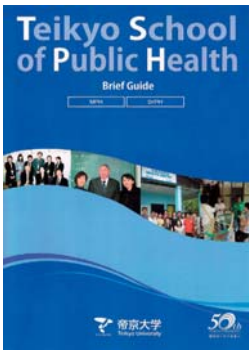
### 5）中央検査部（Central Laboratories）



6）薬剤部（Pharmacy）

## 8．医学部４年生への来年度公衆衛生学実習ガイダンス：ベトナムでの感染症実習

Joining with Medical Students in Teikyo University 4-year's Guidance




## 9．学外：ADC研と連携している結核研究所訪問

Visiting RIT

**The Research Institute of Tuberculosis, Japan（RIT）**

With Dr. Keicho

## 10．冲永佳史学長からの研修修了証授与および歓送会

Certificate Celebration and Farewell Party

**Certificate Award by The President and Farewell Party**

# Reports of Visitors

# SAKURA Science Plan

**Name:** Minh Hang Nguyen
**Country:** Viet Nam
**Department:** Research Biomolecular for Infectious Disease National Hospital of Pediatrics (NHP)
**Position:** Researcher

I am very glad to participate in the Sakura Science Plan. This program is a good opportunity to help me learn and experience a lot for my job. It's very interesting and useful. New technologies and protocols are updated. Teachers are enthusiastic, and the lectures are very interesting. Besides, I also got to visit departments in Teikyo University and Teikyo University Hospital, it has helped me to know more about the working style of Japanese people.

I'm really interested in the technical process applied in the laboratory and the methods to detect virus and bacteria. In addition, I also believe that specific guidelines for biosafety in laboratories are essential. Moreover, the interactions with the staff of Teikyo University and Teykyo University Hospital will be an avenue for building multidisciplinary research collaborations.

**My job in NHP:** Our work in the Biomolecular Laboratory include DNA/RNA isolation, and real-time PCR method for detection of microorganisms. In addition, we also do multiplex real-time PCR to diagnose septicemia in children, involving 25 bacterial species, Luminex to diagnose respiratory tract infection in 18 viruses. Our research is conducted at the laboratory with collaboration in both dosmetic and overseas organizations (e-Asia project, ARDS, SeA…)

**Contribution of this training to my job:** I have updated new technical protocols and learned more about biosafety knowledge, particularly in the laboratory. I have also understood the application of the biosafety guidelines during the tour in ADC. These guidelines will be useful in updating the biosafety manual of my department.

**Collaboration plan with ADC Teikyo University:** I would like to take part in other research projects related to infectious diseases using biomolecular techniques.

**Name:** Nguyen Thi Ngoc Tran, M.D.
**Country:** Hanoi - Vietnam
**Derpartment:** Respiratory Department
National Hospital of Pediatrics
**Position:** Pediatrician & Ph.D. student

It is a pleasure to join with this course. This is a good opportunity for me to improve further the skills, knowledge & experience in a good center in Teikyo University in Tokyo – the capital of Japan. At the same time, I learn experience & culture from Japanese, Filipinos and Lao.

***Job Description:***
Everyday, I examine and treat for patients. There are many patients with acute or chronic respiratory infection in my department. These diseases are caused by virus, bacteria, fungi or parasite. Also, we have other patients who suffer from pleural effusion, pneumothorax, congenital malformation, foreign body of tract respiratory, congenital diaphragmatic hernia, CCAM, pneumonia combined pulmonary artery hypertension, premature pulmonary hypoplasia & etc.

***Benefits from SSP:***
Now, I am a Ph.D student in Military Academy of Medicine. I research infectious factors of severe pneumonia in children due to virus & bacteria, so I need study these content: virus study IL-8 activity by neutrophil chemotaxis, cytokines by qPCR; isolation of RNA/DNA; functional analysis of human neutrophils; biomaker of bacterial infections diseases. This training is helpful to my study.
On the other hand, I can update my knowledge about biosafety, patient safety, biorisk control, pharmacy, epidemiology, molecular techiques for detection of virus, bacteria, TB & fungi to combine the clinic and laboratory. I learned many useful lectures.

***Potential Collaboration:***
If possible, I would like to collaborate with ADC Teikyo University for my study.
(For example: theory, experiment, estimate & public my study results)

**Name:** Doan Thi Mai Thanh, M.D, Ph.D.
**Country:** Vietnam
**Department:** National Hospital of Pediatrics, Hanoi, Vietnam
**Position:** Vice Head of General a Department,
Pediatrician

**Summary of my training course in ADC Teikyo University:**
Biosafety at ADC by Dr. Ito, Biosafety rule by Prof. Suzuki and rounding around Teikyo University Hospital to experience infectious disease control activity in Teikyo University Hospital by Dr. Matsuga were so useful for me. I could listen to lectures on theory and witnessed how Teikyo University Hospital staffs do and coordinate in practical at hospital and laboratory in infection control. It were really good experience. Lecture of Dr. Inoue on Epidemiology was comprehensive. Lecture on clinical support in Africa by Dr. Kato provided us with more knowledge about Ebola virus (guideline, experience in treatment). Lecture of Prof. Shoji Kawachi about ARDS cases due to Influenza Infection in Vietnam was also Interesting. He provided us how to approach with such situation. Rounding around Teikyo University Hospital and university on many different general topics help us to more understanding about your Teikyo University Hospital and Teikyo University. Visiting TB research institute guided by Prof Suzuki and Dr. Ito were so interesting and useful. Prof. Suzuki, Drs. Suzuki and Sugamata taught us some new, useful molecular technique….
**My job in NHP hospital, Vietnam:** I am working at General A Department. My Job is to examine in and out-patient, consult difficulty and severe patients, do some office works, do research, write articles, write lecture…
**Contribution of this training to my job:** Teikyo University Hospital' biosafety practical in hospital were so helpful in my department. I will present such knowledge and experience to my director, head of my department and head of infection control in order to apply it my department and hospital also. Each lectures provided us more knowledge, more understanding what you are doing in research and practical in laboratory and hospital. Rounding around Teikyo University Hospital and Teikyo University in difference topic let us have more understanding about Teikyo University Hospital and Teikyo University, we had opened our eye with such professional thinking and working environment. Even though, I am not lab technicians but learning about some molecular and biology technique and knowledge were also interesting and useful for me: more understanding about technique, knowledge and also give me a passion for scientific research.

**Collaboration plan with ADC Teikyo University**: At present, we received many patients with atypical pneumonia - *M.pneumonia, C.pneumonia* (from 8/2014-9/2015 - we had more 100 patients). And we treated by macrolide as guideline but they did not improve or slowly improved. So we had to change in to Quinolon. We wondered why: the incidence of apical pneumonia resistant to macrolide is so high, why it can atypical bacterial can resistant to macrolid (consider the dose and gen changes...). So we would like to collaborate with Teikyo University to do research on such topic. *H.Influenza, S.pneumonia* also have same situation…

**Name:** Mis Arounnapha Vongdouangchanh
**Country:** Laopeople's Democratic Republic (Lao PDR)
**Department:** National Center for Laboratory
**Position:** Researcher

**We have two Laboratory Section: such Bacteriology Unit and Serology- Virology Unit**
I do work at Bacteriology Unit. That's Job experience.
I work at bacteriology I identify and isolated the causative agents of diseases such as Nosocomial Infection, diarrhea, acute respiratory, pus, body fluid, urine, discharge vaginal…etc.
Molecular typing of pathogenic *E. coli (ETEC, EHEC, EPEC and EIEC).*
PCR testing for *Shigella, Salmonella, Vibrio Cholerae, Plesiomonas shigelloides and Campylobacter*.
Responsible to confirm the bacteria results for hospitals and health centers in the whole country.
Accompany with epidemiology staff to the outbreak area to collect specimens.
Instruct provincial laboratory technician and epidemiology staff to be able to collect specimens, doing rapid test and do some basic testing.
Set up Bacteriology laboratory at the province and district level as required.
Teaching at the faculty of health science.

**Summary of your training in ADC Teikyo University**
The topics for each lecture were mostly beyond my field to study in molecular biology but they all contribute to how we can improve public health using clinical and laboratory methods all lectures made sure the topics were easy to understand and patiently answered all our questions the lecture program was the best and Every Topics very important and very usefully. Some lecture was difficult to follow it seems faraway from my knowledge.

**Contribution of this training to your job**
• Got new Experience, knowledge and high technology from Sakura science plan (SSP)
• Harmonized with Japanese, Vietnamese and Filipino's culture, new knowledge technical laboratory
**Collaboration plan with Asia international Institute of Infectious Disease Control Teikyo university**
• Research cooperation
• Technical exchange

**Name:** Inez Andrea Pastor Medado
**Country:** Philippines
**Department:** Head, Research Section (Science Research Specialist II)
Molecular Biology Laboratory
Research Institute for Tropical Medicine, Philippines
**Position:** Researcher

**Summary of the training**
• The training program covered various disciplines that deal with infectious diseases to improve public health. Each session was taught or facilitated by experts in their respective fields. Sessions consisted of the following:
— Lectures on biosafety, infection control, and infectious disease research using clinical, epidemiological, and laboratory methods.

— Tours of the different departments in the university and hospital and a visit to the Research Institute for Tuberculosis and the JST Miraikan Science Museum.
— Laboratory techniques at ADC: neutrophil chemotaxis, qPCR, PAS strip for molecular detection, and virus plaque assay.
• With two to three sessions every day, the program is light and not difficult to follow. The topics for each lecture were mostly beyond my field of study in molecular biology but they all contribute to how we can improve public health using clinical and laboratory methods. All lecturers made sure the topics were easy to understand and patiently answered all our questions.
• The lectures on infectious diseases were most interesting for me: measles, Influenza H5N1, and tuberculosis. The professors gave comprehensive talks on the clinical, epidemiological, and molecular aspects of the disease.

**Current responsibilities**
— Research on emerging and re-emerging infectious diseases focusing on molecular diagnostic testing and molecular epidemiology.
— Facilitate training programs on molecular techniques for infectious disease diagnostics and research.
— Routine molecular diagnosis for outbreak response to emerging and re-emerging infectious diseases (MERS-CoV, Influenza, Ebola, etc.).

**Contribution of SSP Training to job**
— Adopt new biosafety practices and facility improvements to the laboratory and hospital.
— New perspectives on infectious disease research, especially in coordinating clinical and laboratory methods.

**Collaboration plan with ADC Teikyo University**
— Strengthen current collaboration on cytokine study and develop other research from that project.
— Collaborate on host defense mechanisms of infectious diseases.

**Name:** Hannah Leah E Morito
**Country:** Philippines
**Department:** Molecular Biology Laboratory – RITM
(Science Research Specialist I)
**Position:** Researcher

**Training at Teikyo University**
"The course on training of biosafety and updated molecular techniques for detection of virus and bacteria as conducted by the ADC provided us with several insights, not only in conducting research but also in several aspects of medicine, public health, and clinical healthcare.
"The flow of the schedule also makes the lectures and the exercises we had make a distinctive and lasting impression on us.
"Some of the notable lectures I heard were the lectures in public health and epidemiology, research on environmental fungi in the International Space Station, functional analysis of human neutrophils, and severe ARDS cases due to flu.
"Getting to see the beautiful campus and awesome facilities in the hospital and several laboratories is truly an amazing experience during the first two weeks of our stay in Teikyo University. In our second and third week we had lectures and training workshops in the laboratory, which I found very fascinating and very insightful as well. We were also able to have an opportunity to go out and visit several institutions such as the Miraikan Science Museum and the Japan Anti-Tuberculosis Association in Research Institute for Tuberculosis and learn about their researches."

**CURRENT RESEARCH**
I work as a Science Research Specialist I at the Molecular Biology Laboratory of RITM. I previously worked in serotyping the dengue virus for surveillance using reverse transcription real-time PCR (RT-qPCR) assay and now I am currently working on genotyping the envelope gene of the dengue virus in the Philippines.
I am also involved in molecular diagnosis of emerging infectious diseases such as Influenza and Middle East Respiratory Syndrome (MERS-CoV) using RT-qPCR.

**CONTRIBUTION OF THIS TRAINING**
Almost all of the lectures were notable. For instance, the lecture on severe ARDS cases due to influenza infection in Vietnam got us to thinking of how we can improve the methods that we use in the Philippines to diagnosing cases of flu for immediate treatment and prevention of death.
The lectures also provided us with better understanding of several fields that are not within the scope of our specialties, which

will improve how we respond to situations with public health importance.

**COLLABORATION PLANS**
We should continue strengthening the cytokine research with RITM.
Also, since this training has increased my interest in doing neutrophil and host defense research, despite not having a very strong background on immunology, I think it would be a good idea to explore future collaborations on these areas.

**Name:** Neil Andrew D. Bascos, Ph.D.
**Country:** Republic of the Philippines
**Department and Institution:**
National Institute of Molecular Biology and Biotechnology
University of the Philippines Diliman
**Position:** Assistant Professor
Deputy Director for Facilities and Resources

**SSP Summary:**
I believe that the Sakura Science Plan gives a very *comprehensive* picture of all the different aspects of the capabilities of Teikyo University and its partner institutions (e.g. JATA). The tours of the different facilities, and involvement in the classes and experiments allows participants to experience how it is to be a researcher, student, and member of the Teikyo Team. I greatly appreciate the time and effort that the faculty and staff of Teikyo have devoted to allow us this opportunity, and I hope that this leads to closer ties and collaborative projects between our institutions in the future.

**Job description:**
As Deputy Director for Facilities and Resources of the NIMBB-UPD, I am tasked to oversee the proper enforcement of the rules and guidelines for maintaining Biosafety and Biosecurity in our institute. As an Assistant Professor, I also take charge of different research projects, specifically focusing on protein structure and function.

**Benefits from SSP:**
It was very interesting to see the meticulous way in which these protocols are done here at Teikyo. In particular, I was impressed by the strictness with which access to the restricted areas was controlled at the ADC. The ID monitoring system and the double door locking mechanism (i.e. only one door may be open at a time) may be applied to our own BSL-2 laboratories. The use of a raised platform in the anterooms was also an effective physical reminder for shoe changes. I believe that these protocols are very efficient and we will try to adopt this system in our own institute.
I greatly appreciate the help that the faculty and staff of the ADC have given to allow us to learn new laboratory techniques. I was very fascinated with learning the protocols for measuring chemotaxis and viral plaque assays from Prof. Suzuki' s Laboratory. Our research interests include the analysis of the biophysical characteristics of proteins. We currently study integrins, their heterodimerization and how they may affect metastasis in cancer. I believe we can adapt the protocols for cellular motility to our research and I hope we may collaborate with Prof. Suzuki on this.

**Potential Collaborations:**
**ADC:** *Monitoring effects of integrin heterodimer formation on cancer cell migration*
**Faculty of Pharmacology:** *Structural analysis of Philippine natural products and their target receptors*

ORIGINAL ARTICLE

# Increased γδ$^+$ and double negative T cell subsets in children with bacteremia of *Salmonella oranienburg*: an early diagnostic marker for bacteremia

Kazuhiro Takahashi[1*], Sanpei Miyakawa[2], Kazuya Futatsuyama[2], Atsushi Manabe[3], Hiroshi Shiraga[4], Masakazu Mimaki[1], and Norishi Ueda[5*]

[1]Department of Pediatrics, Teikyo University School of Medicine, Tokyo,
[2]Kidney Center, Tokyo Women's Medical University, Tokyo,
[3]Department of Pediatrics, St. Luke's International Hospital, Tokyo,
[4]Department of Pediatrics, Saiseikai Kurihashi Hospital, Saitama,
[5*]Department of Pediatrics, Public Central Hospital of Matto Ishikawa, Ishikawa, Japan

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## Abstract

**Bacteremia of Salmonella is a risk factor of death in Salmonella infection. Early diagnosis to initiate intensive care is a key for favorable outcome, yet no clinical marker is available to detect bacteremia of Salmonella earlier than blood culture. T, natural killer (NK), and B cells play an important role against Salmonella infection and expansion of γδ$^+$ T cell subset was shown in the study mainly targeting typhoid Salmonella infection by flow cytometry (FCM) which can analyze immune cell populations within two hours.**

**During outbreak of *Salmonella oranienburg* (SO) infection, we explored clinical marker to detect bacteremia in children with or without bacteremia of SO and those with enteritis due to other Salmonella using FCM. We also measured serum concentration of immunoglobulins (Igs) to evaluate whether immunocompromised or not. Eighteen children (median age, 6.0 years) were studied and divided into four groups; group A: five children with bacteremia of SO, group B: the same patients of group A who recovered 3 months after the onset of the disease, group C: six children with enteritis due to SO, and group D: seven children with enteritis due to other Salmonella but no bacteremia. The percentages of γδ$^+$ and double negative (DN: CD4$^-$CD8$^-$) T cells in CD3$^+$ subset were increased in group A as compared to groups B, C and D. There was no difference between groups in the following variables; the percentages of CD3$^+$ cells, helper (CD4$^+$), and cytotoxic (CD8$^+$) T cells, and NK cells, serum levels of Igs or complements. Our data suggest that expanded γδ$^+$ and DN T cell subsets in SO bacteremia by FCM can help to detect SO bacteremia in early stage of the disease faster than blood culture.**

**Key words**: *Salmonella oranienburg*, bacteremia, γδ$^+$ T cells, double negative T cells, flow cytometry

## Introduction

Salmonella is an intracellular parasitic bacteria, and it's infection is a common and usually self-limiting disease[1, 2]. Although *Salmonella oranienburg* (SO) is a relatively uncommon serotype among the non-typhoid Salmonella (NTS)[1], outbreak of SO infection has been reported in several countries, including Japan[3]. Infection of Salmonella causes enteritis and extraintestinal symptoms, including vertebral osteomyelitis, paravertebral or retroperitoneal abscess, and infection of soft tissues and cartilages. Bacteremia occurs in approximately 5-6% of children with NTS infection[1, 2]. While the outbreak of SO infection[3], we experienced five patients with SO bacteremia among 11 patients with SO enteritis and the prevalence of bacteremia was very high (45%) comparing to other NTS infection.

Mortality rate of Salmonella bacteremia is about 12 to 20% in non-immunocompromized patients[4, 5], therefore, early diagnosis and initiation of intensive antimicrobial therapy targeting Salmonella bacteremia is mandatory. Blood culture, the gold standard of diagnosis of bacteremia, usually takes more than 12 hours to detect[6, 7]. Flow cytometry (FCM) is a rapid diagnostic method and predominant activation and expansion of γδ$^+$ T cells in systemic Salmonellosis[8] using FCM suggested diagnostic usefulness for Salmonella bacteremia. Double negative (DN: CD4$^-$CD8$^-$) T cells act as bacteriocidal against infection by intracellular parasitic microorganisms including Mycobacterium infection[9]. Because Salmonella is also an intracellular parasitic microorganism, DN T cells could also play a protective role against Salmonella infection. Thus, we analyzed DN T cell subset as well as that of γδ$^+$ T cells in children with or without bacteremia of SO and those with enteritis due to other Salmonella to test feasibility whether γδ$^+$ and/or DN T cells can predict NTS bacteremia.

From the view of the immune status as a predisposing factor to systemic infection of Salmonella, T cells play an important role for innate immunity. For example, experimental evidence suggests that CD3$^+$ T cells including γδ$^+$ T cells[10, 11] and DN T cells[12] play a protective role for innate immunity to bacterial infection. In vivo and in vitro evidence suggests that CD4$^+$ and CD8$^+$ T cells play a role for immune response to Salmonella infection[13]. In addition, recent in vivo evidence suggests a role

of natural killer (NK) cells[14], antibody production by B cells and complements[15] for early immune response to Salmonella infection. Therefore, we also examined B and NK cell subsets, and serum levels of Igs and complements in this study.

Here we report that increased $\gamma\delta^+$ and DN T cell subsets can predict bacteremia of SO by FCM.

## Material and Methods

*Patient population*

We experienced eleven children with SO infection as previously described[3]. All patients have been healthy until admission to Saiseikai Kurihashi Hospital, Saitama, Japan, and none of the patients had underlying diseases including allergic disease or received immunosuppressive agents. Bacteremia occurred in five of eleven patients (45%) infected with SO and other six patients developed enteritis but no bacteremia. During the same period, we had seven children infected with NTS other than SO who developed enteritis but no bacteremia. These eighteen patients (12 males, 6 females, median age, 6.0 years, range 8 months-15 years) were included in the study. This study has been approved by Ethical Committee in Department of Pediatrics, Saiseikai Kurihashi Hospital and written informed consent was obtained from the parents or guardians of each patient.

Patients were divided into four groups; 1) group A: five patients (median age, 6.0 years) with bacteremia of SO in whom bacteria was detected in both blood and stool cultures, 2) group B: the same patients of group A who had bacteremia but recovered from bacteremia 3 months after the onset of the disease, 3) group C: six patients (median age, 5.5 years) with enteritis in whom SO was only detected by stool culture, and 4) group D: seven patients (median age, 6.0 years) who developed enteritis due to other Salmonella but no bacteremia. Lymphocyte subpopulations vary with age[16-18], however median age at the time of the study was similar between groups.

*Assay for populations of T, NK, and B cells in peripheral blood by FCM*

Peripheral blood mononuclear cells (PBMCs) were obtained from each patient and isolated using Ficoll-Hypaque®. PBMCs of each patient were aliquotted to polystyrene tubes and stained with monoclonal antibodies according to staining panel of subsets (ten subsets) as shown in table 1. Percentage of each subset was analyzed by FACScan® with CellQuest3.3® software as previously described[19]. The data for $CD3^+$, $CD3^+\alpha\beta^+$, $CD3^+\gamma\delta^+$, $CD4^+$, $CD8^+$, and $CD19^+$ cell subsets, except for increased variables, are in compatible with the reference values for lymphocyte subpopulations in age-matched healthy children[16-18].

*Assay for serum levels of immunoglobulins and complements*

Serum levels of IgG, IgA, and complements (C3 and CH50) were measured using laser-nephelometry.

*Statistical analysis*

Data are expressed as median and interquartile range. Comparisons of the data between groups were made using Mann-Whitney U-test. Paired data between groups A and B were analyzed using Wilcoxon matched-pairs signed rank test. A p value less than 0.05 was considered significant.

## Results

There was no difference between groups in the percentages of total $CD3^+$cell subset among total lymphocytes in peripheral blood (Table 1). In contrast, the percentage of $CD3^+\gamma\delta^+$ cell subset in $CD3^+$ cell subset was significantly increased in group A ($p<0.05$) as compared to groups B, C and D. However, it did not differ between groups B, C, and D. Similarly, the percentage of DN T cell subset in $CD3^+$ cell subset was significantly increased in group A ($p<0.05$) as compared to groups B, C and D. It did not differ between groups B, C and D.

There was no difference between groups in the percentages of helper T cell ($CD4^+$) in $CD3^+$ cell subset, activated helper T cell ($CD4^+HLA\text{-}DR^+$) in $CD4^+$ cell subset, cytotoxic T cell ($CD8^+$) in $CD3^+$ cell subset, NK cell ($CD2^+CD56^+$) subset, and B cell ($CD19^+$) subset in peripheral blood. No difference was found between groups in the total lymphocyte count of peripheral blood. In contrast, the percentage of $CD8^+HLA\text{-}DR^+$ T cell subset in $CD8^+$ cell subset was increased in group A as compared to group B and D, but did not differ between group A and C. The percentage of $CD3^+\alpha\beta^+$ cell subset in $CD3^+$ cell subset was decreased in group A as compared to groups C, but there was no difference between groups A, B, and D (table 1).

There was no difference between groups in serum levels of IgG and IgA as well as complements, C3 and CH50.

**Table 1**. Percentages of T cell, NK cell, and B cell subsets in peripheral blood of children with or without bacteremia of *Salmonella oranienburg* and those with enteritis due to other Salmonella

| Variable | Group A (n=5) | Group B (n=5) | Group C (n=6) | Group D (n=7) |
|---|---|---|---|---|
| **T cell** | | | | |
| $CD3^+$ cell in total lymphocyte | 76.0 (6.2) | 73.0 (8.6) | 66.5 (6.0) | 67.0 (12.0) |
| $CD3^+\alpha\beta^+$ cell in $CD3^+$ subset | 70.6 (18.2)* | 81.2 (6.2) | 93.9 (13.0) | 89.3 (4.0)* |
| $CD3^+\gamma\delta^+$ cell in $CD3^+$ subset | 28.7 (17.6)# | 15.6 (4.2) | 10.0 (6.8) | 10.2 (3.8) |
| helper T ($CD4^+$) cell in $CD3^+$ subset | 42.1 (9.2) | 54.5 (15.0) | 54.8 (15.6) | 60.8 (15.0) |
| $HLA\text{-}DR^+$ cell in $CD4^+$ subset | 8.5 (5.0) | 4.3 (1.6) | 4.6 (4.6) | 4.6 (2.0) |
| cytotoxic T ($CD8^+$) cell in $CD3^+$ subset | 33.3 (9.2) | 39.1 (13.2) | 44.3 (12.4) | 39.1 (14.6) |
| $HLA\text{-}DR^+$ cell in $CD8^+$ subset | 27.0 (3.8) | 7.0 (9.4)** | 16.1 (13.0) | 7.7 (12.8)** |
| $CD3^+CD4^-CD8^-$ cell in $CD3^+$ subset | 40.4 (24.6)# | 19.7 (7.6) | 14.9 (12.2) | 13.3 (5.8) |
| **NK cell** | | | | |
| $CD2^+CD56^+$ in total lymphocyte | 9.4 (4.8) | 5.6 (1.6) | 9.6 (4.2) | 7.0 (8.6) |
| **B cell** | | | | |
| $CD19^+$ in total lymphocyte | 11.0 (9.8) | 20.0 (11.6) | 14.5 (9.0) | 18.5 (16.0) |
| Total lymphocytes (cells/μL) | 2,856.6 (3,003.9) | 3,610.0 (2,899.0) | 3,464.6 (4,597.0) | 3,220.6 (1,906.0) |
| **Serum** | | | | |
| IgG (mg/dl) | 1,110.0 (87.5) | 967.0 (137.5) | 1,100.0 (40.0) | 1,000.0 (9.0) |
| IgA (mg/dl) | 180.0 (25.0) | 160.0 (35.0) | 137.0 (12.0) | 150.0 (15.0) |
| C3 (mg/dl) | 110.0 (10.5) | 119.0 (5.5) | 135.5 (6.0) | 116.0 (19.0) |
| CH50 (U/ml) | 44.0 (1.3) | 40.0 (1.5) | 47.0 (3.0) | 44.0 (5.0) |

Percentages of lymphocytes and NK cells in total lymphocytes are expressed in % as median (interquartile).
***** $p<0.05$, vs. Group C only, # $p<0.05$, vs. Groups B, C, and D, ****** $p<0.05$, vs. Group A only.

**Discussion**

Mammalian $CD3^+$ T cells can be separated into two subsets bearing T-cell receptors (TCR), αβ and γδ chains[10]. Most $\alpha\beta^+$ T cells use αβ TCR as antigen recognition[10, 11] and $\gamma\delta^+$ T cells expressing $\gamma\delta^+$ TCR recognize a variety of proteins including pathogen without antigen processing (20). Accumulating evidence suggests that $\alpha\beta^+$ and $\gamma\delta^+$ T cells play a role for innate immunity to bacterial infection[10, 11, 20]. However, little information is available about an alteration of $\alpha\beta^+$ and $\gamma\delta^+$ T cells in NTS infection in humans, in contrast to $\gamma\delta^+$ T cell expansion in bacteremia of typhoid Salmonella[8].

In the present study, the percentage of $\gamma\delta^+$ T cell subset increased at early phase of bacteremia of SO, which retuned to the basal level after recovery of the disease. Our findings have been supported by previous studies documenting increased percentage of $\gamma\delta^+$ T cell subset in children with systemic infection of various strains of Salmonella as compared to those with enteritis[8, 21] although no change in these cells was reported in septic adult patients with Salmonella infection[22]. $\gamma\delta^+$ T cells can be more activated than $\alpha\beta^+$ T cells at early stage of Salmonella infection and expansion of $\gamma\delta^+$ T cells occurs when cultured with live Salmonella typhimurium[8]. Experimental evidence suggests that $\gamma\delta^+$ T cells bind to lipolysaccharides (LPS) through Toll-like receptors, resulting in production of Th-1 like cytokines, including interferon-γ (IFN-γ) or tumor necrosis factor-α (TNF-α) (10, 11), and accumulation of $\gamma\delta^+$ T cells at the site of infection[23]. In fact, $\gamma\delta^+$ T cells have been shown to express higher levels of INF-γ in patients with Salmonella infection than in healthy controls[24]. $\gamma\delta^+$ T cells can activate macrophages, which allows macrophages to produce proinflammatory cytokines that are cytotoxic to bacteria[11]. In addition, in murine model of *Salmonella typhimurium* infection, depletion of $\gamma\delta^+$ T cells can reduce antimicrobial responses such as production of pro-inflammatory cytokines and neutrophil influx in the intestinal mucosa, leading to increased translocation of bacteria to the liver[25, 26]. Our data, along with these in vivo and in vitro data, suggest a role of $CD3^+\gamma\delta^+$ T cells for innate immunity to bacteremia of Salmonella, regardless of strains, in children.

In contrast, the percentage of $\alpha\beta^+$ T cell in $CD3^+$ subset of group A was decreased as compared to groups C and D but it did not differ between groups A and B. These findings suggest a minor value of $\alpha\beta^+$ T cell subset for detection of bacteremia in human NTS infection.

DN T cells play a role for regulation of immune responses in bacterial infection[12]. However, no information is available about an alteration of these T cells in human Salmonella infection. We found increased DN T cell subset in peripheral blood at early phase of bacteremia due to SO, which returned to the basal level after recovery of the disease. Similar increase in the DN T cell subset has been shown in infection with *Leishmania major*[27], *Mycobacterium tuberculosis*, and *Francisella tularensis*[9, 28]. DN T cells can be activated after the recognition of bacterial antigen through the major histocompatibility complex class Ib molecule, CD1b[29]. After being activated, DN T cells can proliferate and regulate immune response by producing Th1 and Th2 type cytokines that activate macrophages[12, 30]. In fact, experimental evidence suggests a protective role of DN T cells in infection due to *Listeria monocytegenes*[30] or malaria[31]. Our data, together with these *in vivo* and *in vitro* data, suggest that DN T cells could play a protective role for innate immunity to bacteremia of SO in humans.

Experimental evidence suggests a role of $CD4^+$ and $CD8^+$ T cells for acquired immunity to Salmonella infection[13]. However, we found no difference in the percentage of $CD4^+$ and $CD8^+$ T cell subsets in PBMCs of patients with or without bacteremia of Salmonella, suggesting a minor predictive value of these cells for bacteremia in human Salmonella infection. The percentage of $CD8^+$HLA-$DR^+$ T cell subset was increased at early phase of bacteremia in our patients as compared to that during recovery phase of the disease and to other NTS. However, it did not differ from that of patients with enteritis due to SO, suggesting a minor predictive value for early diagnosis of SO bacteremia.

NK cells play a role for innate immunity to Salmonella infection[14]. Proliferation and maturation of NK cells, promoted by interleukin-15, can reduce bacterial colonization at intestine and systemic tissues in Salmonella infection[14]. Antibody production by B cells or complement-dependent killing of bacteria[15] also play a role for innate immunity to Salmonella infection. Despite these experimental data, we found no difference between patients with or without bacteremia of Salmonella in the percentages of NK or B cell subset in peripheral blood as well as serum levels of IgG, IgA, C3 or CH50. Our data suggest a minor predicting value of these cells, Igs, or complements for bacteremia in human Salmonella infection.

Leukocytopenia occurs commonly in patients with typhoid fever[32], however, there was no difference in total lymphocytes count in children with or without bacteremia of SO and those with enteritis due to other NTS. This suggests total lymphocyte count has less clinical value for identifying SO bacteremia.

We have analyzed ten subsets of immune cells in PBMCs by FCM and were able to obtain analyzed data sets within two hours using 4 ml of whole blood. According to our result, FCM could distinguish SO bacteremia from SO enteritis prior to obtaining of blood culture. Though not all hospitals can equip this simplest flow cytometer comparing to automated blood culture system, flow cytometer is a powerful tool for investigating immune system to provide quantified data.

In summary, our study showed increased percentages of both $\gamma\delta^+$ and DN T cell subsets in PBMCs at early phase of bacteremia in children infected with SO, which returned to the basal level after recovery of the disease. These data suggest that both $\gamma\delta^+$ and DN T cells could contribute to the prevention of bacteremia in human SO infection and increased population of $\gamma\delta^+$ and DN T cell subsets can predict bacteremia of SO in early phase of the disease by FCM.

**Conflicts of interest: None**

**Acknowledgement**

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**【Current Projects】**

- e-ASIA Joint Research Program: International study on pulmonary diseases infected with influenza virus and *Mycobacterium tuberculosis*
- Roles of NS1 of H5N1 influenza viruses inducing ARDS
- Investigation of the pathogenesis of fulminant pneumonia induced by influenza and development of anti-influenza virus agents
- Innovation of influenza detection kit
- Role of moesin and cytokines of MPO-ANCA associated vasculitis

# INTERNATIONAL MEETING AND SYMPOSIUM

**開催したイベント（2015.6.1 ～ 2015.12.31）**

| 日程 | イベント名 | 演者など |
|---|---|---|
| 2015年11月25日（水） | 11th International Symposium<br>-世界に羽ばたく医療人-<br>【エボラ出血熱】<br>リベリアにおける支援活動から学んだこと | 加藤康幸<br>（国立国際医療研究センター　国際感染症対策室） |
| 2015年11月20日（金） | 10th International Symposium<br>-世界に羽ばたく医療人-<br>【鳥インフルエンザとARDS】<br>ベトナムにおける共同研究で得られた成果 | 河内正治<br>（苫小牧市立病院副院長、国立感染症研究所客員研究員、<br>国立国際医療研究センター麻酔科） |
| 2015年11月19日（木） | 2nd Biosafety Training and Lecture | ADC |
| 2015年11月16日（月）～ 12月4日（金） | さくらサイエンスプラン | ベトナム、フィリピン、ラオスから７名の研修生 |
| 2015年11月9日（月）～ 11月19日（木） | e-ASIA Project Meeting | Annual Meeting |
| 2015年10月10日（土） | e-ASIA Project Meeting | Evaluation by JST |
| 2015年7月16日（木） | バイオセーフティ講習会 | ADC |
| 2015年7月1日（水） | 第9回国際ADC研究所シンポジウム<br>『【アフリカの医療】現実と希望と』 | 木村英作　大阪大学微生物病研究所 特任教授 |

**今後のイベント情報　（2016.1.1 ～ 2016.12.31）**

| 日程 | イベント名 | 演者など |
|---|---|---|
| 2016年10月17日（月）～ 10月18日（火） | e-ASIA Project Meeting | Annual Meeting　Manila, Philippines |
| 2016年9月中　2週間 | Sakura Science Plan No. 2 | ADC |
| 2016年9月21日（水） | 50th Anniversary Symposium | ADC　臨床大講堂 |
| 2016年8月14日（日）～ 8月21日（日） | TASP Training for 6 Students (5-year) | NHP and Bacmai Hospital　Hanoi, Vietnam |
| 2016年5月中 | アジア、アフリカの感染症撲滅 | 大村智、北潔、木村英作、他　臨床大講堂 |
| 2016年5月　前期Ⅲ限 | 危機管理と防災 | 板橋キャンパス危機管理委員会、ADC　臨床大講堂 |
| 2016年1月14日（木） | 第12回国際シンポジウム<br>「福島原発事故後の危機管理と病院の対応」 | 1. 宇野賀津子（ルイ・パストゥール医学研究センター・<br>基礎研究部室長）　大学棟104講義室<br>2. 及川友好（南相馬市立総合病院　副院長）<br>3. 森田知宏（相馬中央病院 内科） |