Annual Report

2014

NCGM-BMH
Medical Collaboration Center

March 2015
Tokyo, Japan-Hanoi, Viet Nam

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Preface

The year 2014 was the fifth year of our intimate collaboration between Bach Mai Hospital (BMH) and the National Center for Global Health and Medicine (NMCG). We, BMH and NCGM, are conducting many activities including the Sister Renal Center Program supported and authorized by International Society of Nephrology started in this year.

This annual report is published to share these results with related authorities and organizations.

We would like to continue our collaboration with both other countries.

Finally, I hope that more fruitful results will be achieved in the next year as well, and I wish that the year 2015 may be good for the people of all over the world, as well as the people of both countries.

Hidechika Akashi, M.D., Ph.D, M.P.H.
Director,
Medical Collaboration Center, in Bach Mai Hospital
National Center for Global Health and Medicine

Abbreviations

BMH	Bach Mai Hospital
NCGM	National Center for Global Health and Medicine
IMCJ	International Medical Center of Japan
MCC	NCGM - BMH Medical Collaboration Center
MOH	Ministry of Health, Viet Nam
MEXT	Ministry of Education, Culture, Sport, Science and Technology, Japan
J-GRID	Japan Initiative for Global Research Network on Infectious Diseases
MHLW	Ministry of Health, Labor and Welfare, Japan
JICA	Japan International Cooperation Agency
MOU	Memorandum of Understanding
HCMC	Ho Chi Minh City
NIHE	National Institute of Hygiene and Epidemiology
NHP	National Hospital of Pediatrics
NLH	National Lung Hospital
HLH	Hanoi Lung Hospital
NIHBT	National Institute of Hematology and Blood Transfusion
RIT-JATA	Research Institute of Tuberculosis-Japan Anti-Tuberculosis Association
WHO	World Health Organization
JFPIMRC	Japan Foundation for the Promotion of International Medical Research Cooperation
SARS	Severe Acute Respiratory Syndrome
DCC	Disease Control and Prevention Center of NCGM

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2. Other activities, topics

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I. General information on NCGM-BMH Medical Collaboration Center (MCC)

1. Background

Since the beginning of 1990's, National Center for Global Health and Medicine (NCGM) (former IMCJ) has been carrying out important roles in collaboration with health sector in Viet Nam for the purpose of the improvement of medical situation in the country. Particularly, collaboration with Bach Mai Hospital (BMH) has been implemented most actively and effectively. In the grant-aid and the technical cooperation projects in BMH, which was supported by Japan International Cooperation Agency (JICA), NCGM contributed to the successful implementation by dispatching experts and providing technical guidance.

Through the history of the past collaboration, NCGM has established close and reliable relationship with BMH and other leading medical institutions in Viet Nam. Using these bases, a new collaboration, which is conducted distinctly from ODA projects and focusing on research and human resource development, was designed.

In order to implement the new collaborative activities, the NCGM-BMH Medical Collaboration Center (MCC) was planed.

2. Establishment of MCC

In view of the successful outcome of BMH project (phase 1) and the efficient collaboration during the SARS outbreak in 2003, a plan to establish a medical collaboration center between NCGM and BMH, which functions separately from JICA projects, grew up in NCGM. The idea was put into practice when the research project on emerging and reemerging infectious diseases was proposed by the Ministry of Education, Culture, Sports, Science and Technology, Japan (MEXT).

In recent years, emerging and reemerging infectious diseases have been threatening the world. In view of the rising fear of these diseases, the MEXT launched a new project in world-wide scale to cope with emerging and reemerging infectious diseases efficiently by setting up medical collaboration centers and conducting close collaboration there. The proposal of the MEXT project facilitated the realization of MCC in Viet Nam. After several preliminary studies, NCGM and BMH decided to establish MCC within BMH based on the friendly and reliable relationship which had been developed since the beginning of 1990's between the 2 medical institutions.

The Memorandum of Understanding (MOU) regarding the initiation of the project was signed by the Director of BMH and the President of NCGM in August 2005 followed by the official approval of the Ministry of Health, Viet Nam. In April 2010, NCGM changed its name (from International Medical Center of Japan; IMCJ to National Center for Global Health and Medicine; NCGM) due to its organizational reform (Independent Administrative Legal Entity). In view of this situation, both sides agreed to revise the MOU along with continuation of the current cooperative activities. After discussions between NCGM and BMH the revised MOU was drafted. In the new MOU, activities in MCC are clarified as research, training, medical case conference, technical cooperation, international conference/ meeting/seminars, personal exchange programs, and others, although in the current version description of activity is concentrated on researches. The new MOU, after getting approval of MOH, was signed by the representatives of BMH (Dr. Nguyen Quoc Anh) and NCGM (Dr. Takaaki Kirino) in June 2, 2010.

MCC office was established in the new building of BMH, which was constructed by Japan's grant-aid in 2000, as the managing center of various collaborative activities including the MEXT project and others. Based on the MCC, various activities were started in collaboration with BMH along with related medical institutions.

Signing ceremony of the Memorandum of Understanding between NCGM and BMH in 2010

3. Objective of MCC

The objective of setting up MCC in Viet Nam is to implement various collaboration on medical science and medical care, such as researches, human resource development & technical exchange, information sharing,

clinical case conference, etc. smoothly and effectively. The activities in MCC are conducted in close collaboration between BMH and NCGM, and related medical institutions and such collaborative activities are expected to benefit both Viet Nam and Japan. The contents of activities can include some advanced and sophisticated techniques which had been difficult to conduct within the framework of JICA projects.

4. Related medical institutions

MCC is mainly collaborating with BMH, however based on the agreement described in MOU; some related medical institutions have been setting up under the approval of BMH.

Currently, five institutions in Hanoi and two institutions in Ho Chi Minh City are functioning as related medical institutions. In the future, more medical institutions might be added if necessary and efficient network building among them of are expected.

Table 1: Related medical institutions of MCC (As of December 2014)

No.	Medical institution	Location	Collaborative study
1	National Institute of Tropical and Infectious Diseases	Hanoi	HIV/AIDS
2	National Hospital of Pediatrics	Hanoi	Clinical conference
3	National Lung Hospital (the former National Hospital of Tuberculosis and Lung Diseases)	Hanoi	Tuberculosis
4	Hanoi Lung Hospital (the former Hanoi Hospital of Tuberculosis and Lung Diseases)	Hanoi	Tuberculosis
5	Ho Chi Minh Medical and Pharmaceutical University	HCMC	Tropical Medicine
6	Ho Chi Minh City Hospital of Tropical Medicine	HCMC	Tropical Medicine Medical education
7	National Institute of Nutrition	Hanoi	Diabetes and life style related disease

5. MEXT project

The Ministry of Education, Culture, Sports, Science and Technology, Japan (MEXT) is implementing the MEXT "Program of Founding Research Centers for Emerging and Reemerging Infectious Diseases" in Asian and African countries. The objective of these activities is to contribute to the emerging infectious diseases and other disease control from the world-wide viewpoint. As of December 2008, this project has been implemented in 11 research centers in 8 countries (Viet Nam, China, India, Thailand, Indonesia, Zambia, Ghana, Philippines). MCC is functioning as one of the important research centers for this project in Viet Nam. The period of this project is 5 years from April 2005 to March 2010. Now, next step starts from April 2010 to March 2015.

Currently, the project supported by MEXT accounts for the major part of the activities of MCC. Activities of the MEXT project in Viet Nam including researches (both basic and clinical researches), human resource development, etc. Equipment which is needed to conduct these activities effectively has also been provided to BMH and relevant medical institutions.

MEXT project in Viet Nam consists of the following three research groups (Dr. Oka is the leader of these researches). These groups are implementing activities on emerging and reemerging infectious disease control based on the concept of MEXT project. The following three researches are three leading research subjects in

MCC and under these research themes, sub-researches have been carried out.

1) Dr. Oka's group: HIV/AIDS
2) Dr. Ohmagari's group: Bacterial infections
3) Dr. Keicho's group: Tuberculosis

6. Non-MEXT projects

In addition to MEXT, the Ministry of Health, Labor and Welfare of Japan (MHLW) is also supporting research projects on various fields. As an oversea base of NCGM, MCC is functioning as a body to support NCGM research teams or individuals who want to implement a collaborative project in Viet Nam.

Within this scope, life-style related diseases such as diabetes and pediatric health issues are projects which have been implemented in collaboration with BMH and other medical institutions of Viet Nam so far. A community-based suvery on diabetes and obesity, followed by an intervention program has been implementing in Hanoi area and a lot of meaningful data have been obtained with the support from MCC.

In 2013, the Sister Renal Center Program was applied in cooperation between Nephro-Urology Department, BMH and Nephrology Department, NCGM to receive the support from the International Society of Nephrology. Under this program, in 2014, a Japanese group including NCGM nephrologists and pathologists came to BMH for a training course on renal biopsy in kidney diseases, with the trainees from BMH and other provincial hospitals. Through this program, the capacity of managing patients with kidney diseases will be increased. Together with the Bureau of International Medical Cooperation of NCGM, MCC participated in supporting the implementation of this program.

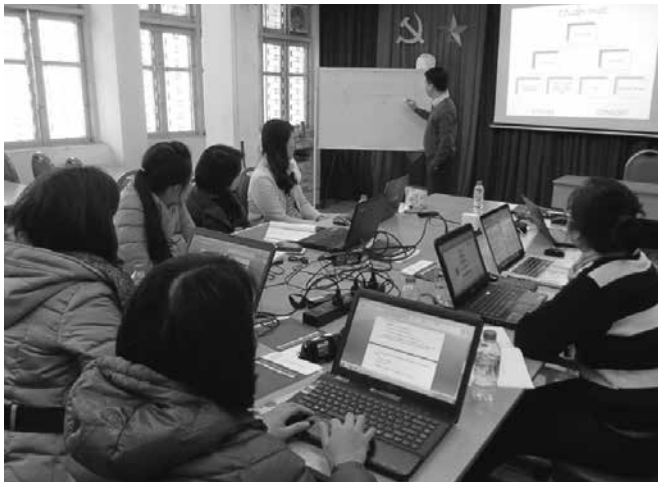
A training course on renal biopsy in kidney diseases held in June 2014
in Bach Mai hospital in collaboration with NCGM doctors

7. Current MCC

In 2014, MCC has received many groups of researchers coming from NCGM and related institutions in Japan. In addition to logistic support for these researchers' groups, such as reservation of accommodation, arrangement of transportation vehicles, on-site coordination including making appointments with Viet Namese counterparts, MCC staff also participated in discussions on research related activities held between the two sides.

In September and October 2014, MCC participated in arrangement of the training visits for NCGM trainees. Groups including doctors, nurses, technicians, and pharmacists came to BMH and other hospitals and healthcare centers in Hoa Binh province for the training on global health and medicine.

Enrichment of knowledge on research support and management is one of the targets for development and sustainability of MCC activities. For this purpose, a series of training courses has been held in MCC since 2010. In 2014, the first session of a four-day course was held in December, with lecturers coming from Hanoi Medical University and participation of all MCC staff including part-time staff. In this course, different topics on research methodology and biomedical statistics were discussed and practiced. Model projects were also conducted for on-the-job training on how to support and manage a collaborative research project.



Training course on research support and management held in December 2014 for MCC staff

II. Activities

1. Research

List of collaborative researches in MCC, Viet Nam

Table 2 Collaborative researches in MCC, Viet Nam

No.	Main Researcher in NCGM	Affiliation in Viet Nam	Subject	Source of fund
1	Shinnichi Oka (AIDS Clinical Center, NCGM)	National Hospital of Tropical Diseases (NHTD)	High treatment retention rate in HIV-infected patients on antiretroviral therapy at two large HIV clinics in Hanoi, Viet Nam	MEXT
2	Shinnichi Oka (AIDS Clinical Center, NCGM)	National Hospital of Tropical Diseases (NHTD)	The cohort study of HIV-1-infected individuals in Northern Viet Nam	MEXT
3	Norio Ohmagari MD., MSc (Director, Disease Control and Prevention Center, NCGM) Nguyen Quoc Anh MD., PhD. (Director, Bach Mai Hospital)	Bach Mai hospital(BMH)	Research on Epidemiology, Diagnosis and Treatment for Healthcare Associated Infection and Antimicrobial Resistant Bacteria in Viet Nam	MEXT
4	Naoto Keicho (Research Institute, NCGM) Luu Thi Lien (Hanoi Department of Health) Pham Huu Thuong (Hanoi Lung Hospital)	Hanoi Lung Hospital (HLH), National Hospital of Lung Hospital (NLH)	Research on tuberculosis in Viet Nam Research on spreading Beijing-genotype strains of *Mycobacterium tuberculosis* and their drug-resistance profiles	J-GRID MEXT
5	Shinsaku Sakurada (Department of RespiratoryDiseases, Research Institute, NCGM)	Medical Collaboration Center-NCGM/Bach Mai Hospital	Study of plasma granulysin and novel biomarkers in HIV tuberculosis (TB) co-infection in Hanoi	J-GRID MEXT
6	Kajio H (Director, Department of Diabetes, Endocrinology and Metabolism, NCGM, Japan) Anh NQ (Director, BMH, Viet Nam) Lien DTK (Director, Department of Diabetes and Endocrinology, BMH, Viet Nam)	Bach Mai hospital (BMH)	Impact of a life style intervention in incident and prevalence of overweight and obesity among secondary school children in Hanoi	1)The Grant of NCGM 2)MHLW 3) Manpei Suzuki Diabetes Foundation
7	Noriko Sato (Department of Pediatrics, National Center for Global Health and Medicine)	National Hue Central Hospital (Hue)	Improvement of short term and long term outcome of childhood encephalitis and/or encephalopathy (E/E) in Viet Nam	MHLW

No.	Main Researcher in NCGM	Affiliation in Viet Nam	Subject	Source of fund
8	Hiroshi Ohara (Bureau of International Cooperation, International Medical Center of Japan)	National Institute of Malariology, Parasitology and Entomology, Bach Mai Hospital	Assessment of the interface between malaria control program and health system strengthening	The Grant of NCGM
9	Naoto Keicho (NCGM/Research Institute of Tuberculosis, JATA) Ngo Quy Chau (Bach Mai Hospital) Le Cong Dinh (Bach Mai Hospital)	Bach Mai hospital	A study on sinopulmonary disease (chronic rhinosinusitis with chronic lower airway infection) in Viet Nam	The Grant of NCGM
10	Naoto Keicho (NCGM/Research Institute of Tuberculosis, JATA)	NCGM-BMH Medical Collaboration Center	Research on establishing basis of international collaborative studies in Viet Nam	The Grant of NCGM
11	Shinsaku Sakurada (Bureau of International Medical Cooperation, NCGM)	Medical Collaboration Center-NCGM/Bach Mai Hospital	To evaluate and improve the training on health care-associated infection control in developing countries	NCGM (25-7)

MEXT: Ministry of Education, Culture, Sports and Science, Japan
MHLW: Ministry of Health, Labor and Welfare, Japan

Research No.1

1.	Title(in English)	High treatment retention rate in HIV-infected patients on antiretroviral therapy at two large HIV clinics in Hanoi, Viet Nam
2.	Title(in Japanese)	ハノイの２つの HIV クリニックにおける治療維持率と治療離脱要因に関する研究
3.	Main researcher	Shoko Matsumoto
4.	Co-Researcher(s)	Junko Tanuma Daisuke Mizushima Nguyen Thi Ngoc Chi Pham Thi Thanh Thuy Do Duy Cuong Nguyen Quang Tuan Nguyen Thi Dung Nguyen Thi Hoai Dung Nguyen Tien Lam Nguyen Van Kinh Shinichi Oka
5.	Resource of fund	Japan Initiative for Global Research Network on Infectious Diseases (J-GRID)
6.	Affiliation(s) in Viet Nam	National Hospital of Tropical Diseases, Hanoi, Viet Nam
7.	Period of the research	From October 2007 to December 2013
8.	Publications	
9.	Summary:	

Background: Loss to follow-up (LTFU) is viewed as a major challenge in improving retention in HIV treatment. In Viet Nam, the reasons for disengagement from clinics and the impact of injection drug use (IDU) on LTFU with unknown outcome (true LTFU) are not well known.

Methods: Patients from two HIV clinics in Hanoi were included in this prospective study between 2007 and 2012, and followed up every 6 months until the end of 2013. The reasons of disengagement from clinic and antiretroviral treatment status during imprisonment were investigated in IDU patients to identify true LTFU. The retention rates at 6-54 months and true LTFU rate were calculated. Cox proportional hazards regression models were performed to identify factors associated with true LTFU.

Results: The study subjects were 1,431 patients, with a follow-up time of 4,371 person-years (median 2.49 years). At the end of the follow-up period, 71 (5.0%) patients died, 79 (5.5%) transferred to other clinics, 16 (1.1%) disengaged from the clinics, and the calculated true LTFU was 45 (3.1%), with 12-month ART retention rate of 95.3% for the entire study population. Imprisonment was the most frequent reason for disengagement from the clinics. True LTFU correlated significantly with low CD4 count and high plasma viral load, but not history of IDU.

Conclusion: Imprisonment seems to be the major cause of disengagement from HIV care among patients with IDU history. However, history of IDU did not correlate with true LTFU.

Research No.2

1.	Title(in English)	The cohort study of HIV-1-infected individuals in Northern Viet Nam
2.	Title(in Japanese)	ハノイにおけるＨＩＶ感染者のコホート研究
3.	Main researcher	Shinichi Oka (AIDS Clinical Center, National Center for Global Health and Medicine)
4.	Co-Researcher(s)	Junko Tanuma, Keiko Saito, Shoko Matsumoto, Nguyen Thi Huyen, Daisuke Mizushima, Fumihide Kanaya, Koji Watanabe, Hiroyuki Gatanaga, Nguyen Hoai Dung, Nguyen Tien Lam, Nguyen Van Kinh, Pham Thi Thanh Thuy, Doan Thu Tra, Vu Thi Tuong Van, Nguyen Quang Tuan
5.	Resource of fund	Ministry of Education, Culture, Sports, Science and Technology of Japan
6.	Affiliation(s) in Viet Nam	National Hospital of Tropical Diseases (NHTD) Bach Mai Hospital (BMH)
7.	Period of the research	October 2007- March 2017
8.	Publications	1. Mizushima et al. ***PLOS One*** 8 (11) e79885, 2013. 2. Tanuma et al. ***JAIDS*** 66 (4): 358-364, 2014. 3. Mizushima et al. ***J Infect Chemothera*** 20 (12) 784-788, 2014.
9.	Summary: Prospective research cohorts of HIV-infected persons have made a major contribution to an understanding of the transmission, natural history and pathogenesis of HIV infection, in addition to generating important information on the response to and long-term outcomes with antiretroviral therapy (ART). Under the project of Japan Initiative for Global Research Network on Infectious Diseases (J-GRID), we have established a hospital-based cohort of HIV-infected individuals in the National Hospital of Tropical Diseases (NHTD) in October 2007 in Hanoi, and the Infectious Disease Department in Bach Mai Hospital has joined the cohort in December 2011, which enables us to follow up patients prospectively with standardized methods of data collection at regular defined time points, for the purpose of clinical researches on HIV/AIDS focused on Asian population. From October 2007 through December 2014, the demographic, clinical and laboratory data had been collected on HIV-positive patients seen at NHTD in Hanoi, Viet Nam. The data collection occurs every 6 months and the data has been stored and managed in a relational database, which was originally created for the East Asia Clinical HIV Cohort (HIV cohort in Japan, Korea and Singapore) and modified for the Hanoi HIV Cohort, enabling us combined analysis of the two cohorts. By the end of December 2014, we recruited 1359 HIV-positive patients on ART and 400 ART-naïve patients in NHTD and 378 HIV-positive patients on ART in BMH to the Hanoi cohort. 63.0% of the cohort was male, and the median age was 32 years at first follow-up. The risk factors for HIV infection were sex between men and women (72.9%) and injection drug use (31.3%). The cohort would provide important information on the status of HIV-infected individuals in Viet Nam and a variety of opportunities to study the unique characteristics on the pathogenesis or the treatment outcome of HIV infection in Asian population.	

Research No.3

1.	Title(in English)	Research on Epidemiology, Diagnosis and Treatment for Healthcare Associated Infection and Antimicrobial Resistant Bacteria in Viet Nam
2.	Title(in Japanese)	ベトナム拠点における医療関連感染症及び後期若耐性菌感染症に関する検討
3.	Main researcher	Norio Ohmagari MD., MSc(Director, Disease Control and Prevention Center, National Center for Global Health and Medicine) Nguyen Quoc Anh MD., PhD. (Director, Bach Mai Hospital)
4.	Co-Researcher(s)	Teruo Kirikae, MD., PhD (NCGM) Tohru Miyoshi-Akiyama, PhD (NCGM) Nozomi Takeshita, MD., PhD (NCGM) Kayoko Hayakawa, MD., PhD (NCGM) Pham Thi Phuong Thuy BA. MPH (NCGM-BMH Medical Coloration Center) Nguyen Gia Binh, MD., PhD Doan Mai Phuong MD., PhD (Head of Microbiology Dept., Bach Mai Hospital) Do Van Thanh (Infectious Dept. and International Dept. Bach Mai Hospital)
5.	Resource of fund	Japan Initiative for Global Research Network on Infectious Diseases (Funded from Ministry of Education, Science and Technology, Culture and Sport of Japan)
6.	Affiliation(s) in Viet Nam	Bach Mai Hospital
7.	Period of the research	April 1, 2012 - March, 2015
8.	Publications	
9.	Summary:	

1. Epidemiology of Healthcare associated infection: The BSI study

Blood culture, which is effective in the diagnosis and treatment of infectious diseases, requires clinical decisions, including the method of examination and evaluation of results. In addition, it has been proven that an appropriate therapy for bacteremia has an impact on patient prognosis. In 2011, the World Health Organization highlighted the importance of health care-associated infectious diseases (HCAI). In middle income countries, the HCAI incidence rate ranges from 5.7% to 19.1%, and notably, the HCAI rates in the intensive care units (ICUs) of these countries are considered to be at least two to three times higher than in high income countries. However, although the surveillance of HCAI is complex, including diagnostic criteria, and sufficient information has not yet been acquired in developing countries, such surveillance is indispensable for implementing countermeasures. In this study, an epidemiological study on the surveillance methods that can be implemented in developing countries is being conducted, with a focus on catheter-related infections among HCAI using blood culture surveillance, which is a basic diagnosis of severe infection, and through epidemiological information in Viet Nam. Note that this study has already been started, internally. A comparison of the information on infectious diseases between countries is also being conducted, by comparing the relevant study information with that in Japan, and examinations will also be conducted to clarify issues regarding infection control.

As of Dec 2014, we had already finished the phase I study, retrospective analysis of blood culture isolates at Bach Mai Hospital, and that has already submitted for publication. The phase II study, which is the retrospective observation study for bloodstream infection in ICU , has already started and >50 cases were registered as of the end of Dec, 2014.

(Abstract for phase I study)

Assessment of Bacteremia in a Large Tertiary Care Hospital in Northern Viet Nam: A Single-center Retrospective Surveillance Study

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Bacteremia is one of the major causes of morbidity and mortality. The clinical analysis of Bacteremia cases would be valuable for its diagnosis, treatment, and prevention.

However, thus far, limited data on bacteremia are available in Viet Nam. A single-center, retrospective, surveillance study was conducted in Bach Mai Hospital, Hanoi, Viet Nam from 2009 to 2012. In total, 45,366 blood culture cases were analyzed. The number of blood cultures per 1000 patient-days was 9.59 sets. The percentage of solitary blood culture sets was 49.6%. The rate of positive blood culture was 13.9%. The major pathogens isolated in adults were coagulase-negative Staphylococcus species (16.7%), followed by Escherichia coli (6.8%), Streptococcus spp. without Streptococcus pneumonia (3.8%), and Staphylococcus aureus (5.2%). Other major pathogens were Klebsiella spp. (4.2%), Salmonella spp. without typhi and paratyphi A (1.7%), Acinetobacter spp. (2.2%), Candida spp. (2.1%), and Burkholderia spp. (4.0%). It is difficult to judge the Viet Nam data appropriately on its own; therefore, we compared it with the results of a multicenter study of Japanese hospitals. The number of blood cultures per 1000 patient-days was lower and the percentage of solitary blood culture sets in the present study was higher than that of the Japanese study (9.6 vs. 25.2 and 49.6% vs. 32.8%, respectively). Particularly, the distribution of microorganisms, which may differ by country, was unique in regard to Acinetobacter and Salmonella cases. The rate of bacteremia microorganisms was different from that reported in previous studies, especially, that of Salmonella typhi. The percentage of bacteremia cases caused by healthcare-associated infection may be relatively high.

2. Molecular epidemiology of multidrug-resistant Gram-negative pathogens based on their whole genome sequencing

Study Facility: Bach Mai Hospital, Hanoi, Viet Nam
National Center for Global Health and Medicine, Tokyo, Japan

Researchers :

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1. Background

The emergence of multidrug-resistant (MDR) Gram-negative pathogens, such as MDR Pseudomonas aeruginosa and *Acinetobacter baumannii*, has become global concern, since they has been acquired to highly resistance to almost all β-lactam antibiotics, including penicillins, cephalosporins and carbapenems.

MBLs produced by gram-negative bacteria, including *Pseudomonas aeruginosa*, *Acinetobacter* spp, and other *Enterobacteria*, confer resistance against all β-lacatams except for the monobactams. Based on amino acid

equences, MBLs are categorized into various types including IMP, GIM, SPM, SIM, VIM, KHM and NDM types. The IMP- and VIM-type enzymes are the most common and exhibit a worldwide distribution. Recently, SPM, GIM, SIM and KHM have been reported from Brazil, Germany, Korea and Japan, respectively. More recently, NDM-1 have been reported from India, Pakistan and European countries as well as Japan.

Next generation sequencers (NGSs) have brought paradigm shift in the field of microbiology. The most advanced NGS system can generate 1Tbp sequence data in about a week, which is equivalent to 200,000-folds of average bacterial genome size. Using multiplexing technologies, it is possible to analyze more than hundreds of isolates at once. With the aid of high performance workstations and bioinformatics analyses, we can analyze the whole genome sequence data of each isolate at a SNP level, which allow us to distinguish an outbreak cases from the sporadic cases. NGSs and the data analysis would be indispensable.

The objectives of this study are to detect carbapenemase- and 16S rRNA methylase-producing isolates of Gram-negative pathogens in Bach Mai Hospital, to determine the genetic and phenotypic properties of the carbapenemases and 16S rRNA methylases produced by these clinical isolates, and introduce PCR diagnostic methods for detection of MBLs-producing isolates. The study was designed as followed;

1.1 To screen clinical samples obtained from patients in wards with high risks of nosocomial infections for MDR gram-negative bacteria.

1.2 To determine the minimal inhibition concentrations (MICs) of carbapenems and aminoglycosides.

1.3 To screen the isolates for detection of genes encoding carbapenemases and 16S rRNA methylases with next generation sequencers (NGSs).

We previously did a collaboration study with Dr. Nguyen Viet Hung and Dr. Doan Mai Phuong(Bach Mai Hospital) on drug-resistant A. baumannii and P. aeruginosa isolates obtain from patients in the intensive care unit in Bach Mai Hospital (1). As the results, a majority of these isolates (more than 70%) were highly resistant to amikacin, arbekacin and gentamycin with the MICs of more than 1,024 mg/L. The collaboration study revealed that they were producing 16S rRNA methylases, ArmA and RmtB. Aminoglycosides have a high affinity for the 16S rRNA of the bacterial 30S ribosome and block protein synthesis. The methylation of 16S rRNA makes these bacteria highly resistant to all clinically important aminoglycosides. Clinical isolates of highly aminoglycoside-resistant Gram-negative bacteria producing16S rRNA methylase were identified in France and Japan. Since then, 16S rRNA methylase-producing Gram-negative bacteria have been isolated in other parts of the world, including Asian countries such as Afghanistan, Bangladesh, China, Hong Kong, India, Japan, Korea, Oman and Pakistan. This was the first report describing the presence of methylase producing Gram-negative bacteria in medical settings in Viet Nam (1).

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3. The low incidence of nephrotoxicity associated with colistin use in intensive care unit in Viet Nam: Tailored use of colistin in a population with low body weight

Nguyen Gia Binh[1], Kayoko Hayakawa[2], Dao Xuan Co[1], Nguyen Dang Tuan[1], Nguyen Hoang Anh[3], Nguyen Thi Hoang Thuy[4], Doan Mai Phuong[5], Nguyen Thi Lien Huong[3], Pham Thi Phuong Thuy[6], Ngo Qui Chau[7], Pham Hong Nhung[5], Do Thi Hong Gam[4], Duong Thanh Hai[4], Tran Thu Huong[4], Le Van Anh[4], Nozomi Takeshita[2], Norio Ohmagari[2]

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Objectives:

Multi-drug resistant Gram-negative bacteria (MDR-GNB) have spread worldwide, including Asia. Colistin is a key drug for treating infections caused by MDR-GNB. However, data are scarce regarding the incidence of nephrotoxicity, efficacy, and optimal dosing of colistin in the Asian population.

Methods:

This was a retrospective observational study to assess the efficacy and nephrotoxicity of intravenous colistin in critically ill patients treated at Bach Mai Hospital between August 15, 2013 and January 15, 2014. Bach Mai Hospital has 2,000 beds, and serves as a tertiary care hospital in Hanoi, Viet Nam. Adult patients aged >18 years were included in this study if they were admitted to the intensive care unit and received intravenous colistin for a hospital-acquired MDR-GNB infection, as confirmed by a positive microbiological culture. Colistin was administered according to dosing suggestions that were based on pharmacokinetic/pharmacodynamic and toxicodynamic principles. The loading dose (colistin base activity, mg) was defined as: Ctarget × 2 × total body weight (kg), and the maintenance dose was defined as: Ctarget × (1.5 × creatinine clearance rate [mL/min] + 30).

Results:

During the study period, 28 eligible patients were identified. The mean patient age was 60 ± 20.4 years, and 18 (64%) were men. The mean body weight for the study cohort was 53 ± 8.6 kg (range, 35.5–75 kg). Eight (28.6%) patients had preexisting renal failure prior to the administration of colistin, and the majority (n = 26; 92.9%) of patients had ventilator-associated pneumonia. Acinetobacter baumannii was the most frequently isolated bacteria (n = 24; 85.7%), followed by Pseudomonas aeruginosa (n = 3; 10.7%) and Klebsiella pneumoniae (n = 3; 10.7%). The minimum inhibitory concentrations (MIC50 and MIC90) of colistin for A. baumannii were 0.125 mg/L and 0.75 mg/L,

respectively. The mean dose of colistin used in this study was 4.1 ± 1.6 million IU. The mean duration of colistin therapy was 12.5 ± 5.2 days, and the mean cumulative dose of colistin was 48.2 ± 22.8 million IU. In all cases, colistin was used as a combination therapy, with either carbapenem (n = 25; 89.3%), a beta-lactam/beta-lactamase inhibitor (n = 3; 10.7%), or carbapenem and fluoroquinolone (n = 3; 10.7%).

Colistin therapies were classified as “responsive” in 19 (67.9%) cases, or as “unresponsive” in 9 (32.1%) cases, based on the clinical and microbiological assessment. Five (17.9%) patients who did not respond to colistin died; 6 (21.4%) patient developed nephrotoxicity during the study period per RIFLE criteria.

Conclusion:

A tailored dosing protocol of colistin was effective, with low nephrotoxicity, among critically ill Viet Namese patients with low body weight compared to the participants in previous studies. Further studies are warranted for assessing the efficacy and toxicity in a larger cohort.

Educational activities:

Training Course on Case Management of Tropical Infectious Diseases was held at Ho Chi Minh City, Viet Nam (December 2013). Three infectious diseases residents and one fellow from NCGM had participated in this training course.

Research No.4

1.	Title(in English)	Research on tuberculosis in Viet Nam Research on spreading Beijing-genotype strains of *Mycobacterium tuberculosis* and their drug-resistance profiles
2.	Title(in Japanese)	ベトナムにおける結核症に関する研究 結核菌北京型株の蔓延と多剤耐性に関わる研究
3.	Main researcher	Naoto Keicho (NCGM/Research Institute of Tuberculosis, JATA) Luu Thi Lien (Hanoi Department of Health) Pham Huu Thuong (Hanoi Lung Hospital)
4.	Co-Researcher(s)	Vu Cao Cuong (Hanoi Lung Hospital) Nguyen Van Hung (National Lung Hospital) Shinji Maeda (Research Institute of Tuberculosis, JATA) Minako Hijikata (NCGM/Research Institute of Tuberculosis, JATA) Nguyen Thi Le Hang (NCGM-BMH Medical Collaboration Center) Shinsaku Sakurada (International Bureau, NCGM)
5.	Resource of fund	the Program of Japan Initiative for Global Research Network on Infectious Diseases (J-GRID), MEXT
6.	Affiliation(s) in Viet Nam	Hanoi Lung Hospital (HLH), Viet Nam National Lung Hospital (NLH), Viet Nam
7.	Period of the research	2010-2014
8.	Publications	1. Hijikata M, Matsushita I, Hang NT, Maeda S, Thuong PH, Tam do B, Shimbo T, Sakurada S, Cuong VC, Lien LT, Keicho N. Age-dependent association of mannose-binding lectin polymorphisms with the development of pulmonary tuberculosis in Viet Nam. **Hum Immunol**. 2014;75(8):840-6. 2. Maeda S, Hang NT, Lien LT, Thuong PH, Hung NV, Hoang NP, Cuong VC, Hijikata M, Sakurada S, Keicho N. *Mycobacterium tuberculosis* strains spreading in Hanoi, Viet Nam: Beijing sublineages, genotypes, drug susceptibility patterns, and host factors. **Tuberculosis** (Edinb). 2014;94(6):649-56. 3. Hang NT, Matsushita I, Shimbo T, Hong le T, Tam do B, Lien LT, Thuong PH, Cuong VC, Hijikata M, Kobayashi N, Sakurada S, Higuchi K, Harada N, Endo H, Keicho N. Association between tuberculosis recurrence and interferon-γ response during treatment. **J Infect**. 2014;69(6):616-26.
9.	Summary: **Overall purpose** • To strengthen collaborative research work on tuberculosis (TB) between Viet Nam and Japan • To prevent generation and spread of drug-resistant TB and TB-HIV co-infection **Output** A. NCGM-RIT-HLH collaboration	

	1. Analysis of Hanoi-TB data containing clinical, genome-epidemiological, immunological and bacteriological information, and specimens. 2. Improvement of diagnosis, monitoring, treatment and prevention of TB and understanding process of TB infection and development. 3. Identification and reduction of risk factors to prevent spread of drug-resistant TB and TB-HIV co-infection. B. NCGM-RIT-NLH collaboration 1. Genome epidemiology of *Mycobacterium tuberculosis* (MTB) strains in Hanoi. 2. Analysis of drug-resistant MTB. 3. Analysis of reactivation and re-infection of MTB after anti-TB treatment.

Research No.5

1.	Title(in English)	Study of Plasma granulysin in HIV tuberculosis (TB) co-infection
2.	Title(in Japanese)	HIV 結核共感染における血漿グラニュリシンの研究
3.	Main researcher	Shinsaku Sakurada (Bureau of International Medical Cooperation, NCGM)
4.	Co-Researcher(s)	Nguyen Thi Le Hang (MCC), Le Xuan Hai (NIHBT), Pham Huu Thuong (HLH), Do Bang Tam (HLH), Minako Hijikata (JATA-RIT), Naoto Keicho (JATA-RIT)
5.	Resource of fund	J-GRID/MEXT
6.	Affiliation(s) in Viet Nam	Medical Collaboration Center-NCGM/Bach Mai Hospital
7.	Period of the research	April, 2010-March, 2015
8.	Publications	Poster Presentation: S Sakurada, P T M Ngoc, N T L Hang, D B Tam, L T Hong, V C Cuong, P H Thuong, T Tanaka, N Keicho, Granulysin in HIV/TB coinfection and latent TB. Asia-Africa Research Forum in Yokohama, January 2013.
9.	Summary: Granulysin is a saposin family protein released from NK cells, NKT cells, γδ T cells and cytotoxic T cells (CTL). This molecule induces apoptosis of target cells working with perforin and has significant bacteriocidal activity with broad spectrum. Precursor and mature protein has 15kDa and 9kDa molecular weight respectively. Mature protein co-exists with perforin and granzymesin cytotoxic granules and is released from the effector cells by degranulation mechanism. Precursor protein has significant chemotactic activity for mononuclear blood cells. Levels of plasma granulysin in patients with active TB are significantly lower than those in healthy individuals and change of the plasma levels during anti-TB chemotherapy is associated with clinical outcome. To study possible roles of granulysin in HIV/TB co-infection, we started a clinical study in Hanoi by recruiting healthy donors as a control group and patients with single infection of HIV and TB, and HIV/TB co-infection in 2012. Blood samples were collected only once in healthy donors and the patients with HIV, twice at the introduction and end of anti-TB treatment in the patients with TB, three times, at the introduction of anti-TB treatment and ART, and at the end of anti-TB treatment in the patients with HIV/TB co-infection. The levels of granulysin in plasma were determined using ELISA and the cellular expression and distribution of granulysin in peripheral blood mononuclear cells were analyzed by flow cytometry using anti-granulysin monoclonal antibody. We will analyze the association between plasma granulysin and clinical manifestation in the patients with HIV/TB co-infection. By the mid of 2014 we had recruited patients more than 70% of them in the research plan. It will be continuously conducted by the end of March 2015. Separately we conducted studying the association between plasma granulysin and result of interferon-γ release assay (IGRA) in healthy population, since role of granulysin in latent TB infection has not been fully understood in 2012. Additionally we analyzed SNPs in granulysin gene. To publish the report regarding this study we prepare a daft paper.	

Research No.6

1.	Title(in English)	Impact of a life style intervention in incident and prevalence of overweight and obesity among secondary school children in Hanoi
2.	Title(in Japanese)	ハノイ市の中学生における肥満・過体重に対する生活習慣介入に関する研究
3.	Main researcher	Kajio H (Director, Department of Diabetes, Endocrinology and Metabolism, NCGM, Japan) Anh NQ (Director, BMH, Viet Nam) Lien DTK (Director, Department of Diabetes and Endocrinology, BMH, Viet Nam)
4.	Co-Researcher(s)	JAPAN: Matsushita Y (NCGM), Kishimoto M (NCGM), Tsujimoto T(NCGM), Hara M (Tokyo Metropolitan Hiroo General Hospital) Viet Nam: Thanh DVT (BMH), Thanh NTT (BMH), Thuy PTP (MCC)
5.	Resource of fund	1) The Grant of National Center for Global Health and Medicine, NCGM, Japan 2) The Grant of Ministry of Health, Labor and Welfare of Japan 3) Manpei Suzuki Diabetes Foundation
6.	Affiliation(s) in Viet Nam	Bach Mai Hospital
7.	Period of the research	Dec 2012 -
8.	Publications	In Preparation
9.	Summary: Overweight and obesity lead to adverse metabolic effects on blood pressure, cholesterol, triglycerides and insulin resistance. Risks of coronary heart disease, ischemic stroke and type 2 diabetes mellitus increase steadily with increasing body mass index (BMI). Overweight and obese children are likely to stay obese into adulthood and more likely to develop non-communicable diseases (NCD) like diabetes and cardiovascular diseases at a younger age. In the developing countries, nowadays, the increasing prevalence and incidence of overweight and obesity is a serious public health problem following the social and economic development of the country. The prevalence has increased at an alarming rate even in children. Our aim of this study is to study the impact of a life style intervention in incident and prevalence of overweight and obesity among secondary school children in Hanoi from 2012 to 2015. **Goal of the theme:** Overall goal: Reduce prevalence and incident in secondary school children. **Specific goals:** 1) To find whether length of education had an impact on prevalence and incident of overweight and obesity in the children. 2) To evaluate the impact of the intervention to lifestyle changes in dietary control, physical exercise among the children. 3) To improve the knowledge of overweight and obesity and prevention against lifestyle-related diseases in the secondary school children.	

Methods:

We started an intervention study with controlled group to see longitudinal changes of anthropometrical indices, diet and intergraded physical activities, lifestyle and biomarkers such as FBG, HbA1c, insulin level, lipid profile, and adiponectin after the baseline surveillance in Dec, 2013 and in Jan, 2014.

We recruited 821 children of 6th grade from 4 different schools (Cat Linh school, Nguyen Cong Tru School, Phan Chu Trinh School, Dong Da school) , which were randomly selected from 2 urban districts in Hanoi. The information of the baseline surveillance included the lifestyle conditions and the knowledge levels of the children as well as the parents provided by the questionnaires.

After allocation of 4 schools into two groups, two schools for the intervention group and the other two schools for the control group, we have been performing intervention activities. The intervention activities are integrated by four components; physical activity, nutritional, lifestyle behavior change and wide communication tools. We provided the participants with the pedometers, and the participants in the intervention group were also provided with the scales. As for the behavior intervention, we set up several targets to promote the participants in the intervention group to continue the activities with self-monitoring, goal setting, and problem solving.

The 1-year mid-term surveillance was performed in Dec, 2013 and in Jan, 2014. The meetings for the mid-term evaluation will be scheduled in Mar, 1995.

Results:

We obtained the baseline data from 821 children of 4 schools. We found that the prevalences of children with underweight, normal-weight, overweight and obesity were 4.1%, 59.7%, 16.9% and 19.2%, respectively, following WHO standard cut-offs. Multiple logistic regression analysis to predict risk factors for overweight/obesity (OW/OB) after adjustment with sex, the OR of OW/OB for children is increased by paternal OW/OB (reference: BMI 18.5-24.9 kg/m2; BMI≥25: 2.02, p=0.001), maternal OW/OB (reference: BMI 18.5-24.9 kg/m2; BMI≥25: 2.831, p=0.001), parental OV/OB (reference: parental no OW/OB, father or mother OW/OB: 2.22, $p < 0.001$; both of parental OW/OB: 6.59, $p = 0.024$), increased birth weight (reference: 2500-3500g; ≥ 3500g: 1.52, $p = 0.02$), less sleeping hour per day (reference: < 8 hours; 8 -11: 0.57, $p < 0.001$; >11:0.44, $p = 0.02$), less physical activities to lose weight (reference: no, yes : 6.185, $p < 0.001$), less food to lose weight (reference: no, yes : 8,239, $p < 0.001$), and more vegetable to lose weight (reference: no, yes : 3.908, $p < 0.001$).

The data for the 1-year mid-term surveillance is now under analysis.

Discussions and Conclusions:

We identified the higher prevalence of overweight or obesity in school children. We also clarified the importance factors influencing on the appearance of overweight or obesity. These factors include family anthropometrical factors as well as several lifestyle factors such as sleeping duration, food and physical activities. We are now continuing the intervention trial to identify the impact of lifestyle intervention on prevalence and incident of overweight and obesity in the children.

Research No.7

1.	Title(in English)	Improvement of short term and long term outcome of childhood encephalitis and/or encephalopathy (E/E) in Viet Nam.
2.	Title(in Japanese)	国際医療研究開発事業 (24 指 109)：ベトナムにおける小児脳炎・脳症の短期及び長期予後の改善に関する研究
3.	Main researcher	Noriko Sato (Department of Pediatrics, National Center for Global Health and Medicine)
4.	Co-Researcher(s)	Noriko Sato, Takeji Matsushita, Hideko Uryu,
5.	Resource of fund	International Health Research (A24-109) from Ministry of Health Labor and Welfare of Japan
6.	Affiliation(s) in Viet Nam	National Hue Central Hospital (Hue)
7.	Period of the research	April 2012- March 2015
8.	Publications	

9. Summary:

Background:

This study is intended to achieve better understanding of clinical state and improving outcome of E/E at the pediatric department in Hue Central Hospital Hue, Viet Nam.

Methods:

The research protocol consists of two studies; one is retrospective case-control study, and another is prospective study. The statistical data about E/E at Hue Central Hospital is extracted retrospectively from medical record of patients for three years. And in prospective study part, clinical status, especially etiologic agent and treatment of E/E patients will be explored.

Results:

The clinical data obtained in Hue Central Hospital between 2009 and 2011 were retrospectively analyzed. Although some of the data were missing and we are currently enquiring the updated data from Hue, the following pilot results are represented. In 3 years, approximately about 100 patients were diagnosed as E/E; however, 62 out of them had not been examined to identify the pathogen. In cases of the pathogen were identified, Rubella encephalitis is the most common, followed by Japanese encephalitis. Although the serological confirmation was not available, encephalitis associated with hand-foot-and-mouth disease (HFMD) based on the clinical observation/diagnosis was also common reflecting the epidemic situation of all around Viet Nam. In the case of HFMD, neurological findings and prognosis are also described and further retrospective analysis is expecting.

On the other hand, the prospective research project of E/E has been started and over 60s patients were enrolled to date. Serum and cerebrospinal fluid samples were collected and sent to National Institute of Infection Diseases, Japan to detect the antibodies against various pathogens of E/E. We are now analyzing these samples and data to make clear the situation of E/E in Hue.

Research No.8

1.	Title(in English)	Assessment of the interface between malaria control program and health system strengthening
2.	Title(in Japanese)	マラリア対策とヘルスシステム強化に関する研究
3.	Main researcher	Hiroshi Ohara (Bureau of International Cooperation, International Medical Center of Japan)
4.	Co-Researcher(s)	Vu Huy Nam (Dep. of Planning, National Institute of Malariology, Parasitology and Entomology) Pham Thi Thanh Thuy (Dept. of Infectious Diseases, Bach Mai Hospital) Jeevan B. Sherchand (Dept. of Public Health, Instititute of Medicine, Tribhuvan University, Nepal)
5.	Resource of fund	Grants of National Center for Global health and Medicine (24-5), Japan.
6.	Affiliation(s) in Viet Nam	National Institute of Malariology, Parasitology and Entomology, Bach Mai Hospital
7.	Period of the research	October 2012- March 2015
8.	Publications	Oral presentation 29th Annual Meeting of Japan Association for International Health (November 2014, Tokyo, Japan)
9.	Summary: **Study in Viet Nam:** In Viet Nam malaria has been a major health issue with high priority in control strategy. However due to the great efforts of the government to implement effective control measures, malaria has been successfully controlled. In this study, successful factors in malaria control were analyzed, particularly in relation to health system. Information obtained from the document reviews, interviews, and field surveys were analyzed from the viewpoint of interface between malaria control program and the health system in accordance with the 6 building blocks of a health system (Leadership and Governance, Service delivery, Workforce, Information system, Medical products and technology, and Financing) with special emphasis on good practices and challenges in implementing malaria control program. **Leading good practices include:** 1)Strong government commitment for malaria control, 2)National strategy for rural development and intensified education for residents, 3)Effective vertical system from national to village level for malaria surveillance and service delivery, 4)Domestic antimalarial production and high coverage of control measures, 5)Strengthening the capacity of health workers along with mobilization of mass organizations, and 6) Support from international organizations. **Key challenges include:** 1)Large number of population at risk in remote and mountainous areas, 2)Uncontrolled seasonal migrants and cross border movement, 3)Lack of human resources for malaria network, and 4)Increasing drug resistance of malaria parasite. Effective implementation under the strong leadership of National Steering Committee utilizing the existing health system was outstanding. Besides, strengthening of the vertical health program appeared to have a good impact on the general health system, particularly at the primary level.	

	Study in Nepal and comparison with Viet Nam: Malaria control has been a major health issue with high priority in endemic countries. In this study, successful factors in malaria control and key challenges were analyzed, particularly in relation to health system. Studies were conducted in Nepal and Viet Nam. Information obtained from the document reviews, interviews, and field surveys were analyzed from the viewpoint of interface between malaria control program (vertical health system) and the general health system with special emphasis on good practices and key challenges. **Leading good practices include:** 1)Strong government commitment for malaria control (Particularly in Viet Nam), 2)National strategy for rural development and intensified education for residents, 3)Effective vertical system from national to village level for malaria surveillance and service delivery, 5)Strengthening the capacity of health workers along with mobilization of mass organizations, and 6)Support from international organizations. **Key challenges include:** 1)Large number of population at risk in remote and mountainous areas, 2)Uncontrolled seasonal migrants and cross border movement, 3)Lack of human resources for malaria network, and 4)Increasing drug resistance of malaria parasite, 5)Inequality in distribution of bed nets (Particularly in Nepal). Strengthening of the vertical health program appeared to have some impact on the general health system, particularly at the primary level. More efforts are requested for strengthening of the health system in remote areas, training of health staff at peripheral level, accurate quality assurance, promotion of public-private relationship and equity in bed net distribution. These tackling will directly lead to further strengthening of the general health system, and eventually effective implementation of various health programs and contribution to UHC.

Research No.9

1.	Title(in English)	A study on sinopulmonary disease (chronic rhinosinusitis with chronic lower airway infection) in Viet Nam
2.	Title(in Japanese)	ベトナムにおける副鼻腔気管支症候群の研究 (25 指 5 の分担研究として)
3.	Main researcher	Naoto Keicho (NCGM/Research Institute of Tuberculosis, JATA) Ngo Quy Chau (Bach Mai Hospital) Le Cong Dinh (Bach Mai Hospital)
4.	Co-Researcher(s)	Minako Hijikata (NCGM/Research Institute of Tuberculosis, JATA) Yuichi Majima (Ise Municipal General Hospital) Pham Minh Thong (Bach Mai Hospital) Pham Thien Ngoc (Bach Mai Hospital) Phan Thu Phuong (Bach Mai Hospital) Le Thi Tram (Bach Mai Hospital) Nguyen Thi Le Hang (NCGM-BMH Medical Collaboration Center) Pham Thi Ngoc Bich (NCGM-BMH Medical Collaboration Center)
5.	Resource of fund	a grant of National Center for Global Health and Medicine
6.	Affiliation(s) in Viet Nam	Bach Mai hospital
7.	Period of the research	2013-2015
8.	Publications	
9.	Summary: **Overall purpose** • To identify host genetic factors involved in development of sinopulmonary disease in Viet Nam • To characterize clinical background of sinopulmonary disease in Viet Nam • To investigate prevalence and risk factors for chronic lower respiratory infection among patients with chronic rhinosinusitis in Viet Nam	

Research No.10

1.	Title(in English)	Research on establishing basis of international collaborative studies in Viet Nam
2.	Title(in Japanese)	ベトナム海外拠点における高品質な臨床疫学研究の実施と支援体制の整備に関する研究　(25 指 5 の分担研究として)
3.	Main researcher	Naoto Keicho (NCGM/Research Institute of Tuberculosis, JATA)
4.	Co-Researcher(s)	Hoang Van Minh (Hanoi Medical University)
5.	Resource of fund	a grant of National Center for Global Health and Medicine
6.	Affiliation(s) in Viet Nam	NCGM-BMH Medical Collaboration Center
7.	Period of the research	2013-2015
8.	Publications	
9.	Summary: **Overall purpose** To strengthen skills of collaborative research in NCGM-BMH Medical Collaboration Center **Activities** 1. Lecture training on skills of implementation of clinical research 2. On the job training as research associates for model research	

Research No.11

1.	Title(in English)	To evaluate and improve the training on health care-associated infection control in developing countries
2.	Title(in Japanese)	開発途上国における院内感染対策研修の評価と改善
3.	Main researcher	Shinsaku Sakurada (Bureau of International Medical Cooperation, NCGM)
4.	Co-Researcher(s)	Toru Akiyama (University of Tsukuba), Yumiko Haneishi (NCGM), Nozomi Takeshita (NCGM), Narumi Hori (NCGM), Hiroshi Ohara (NCGM), Dai Yoshizawa (NCGM)
5.	Resource of fund	NCGM (25-7)
6.	Affiliation(s) in Viet Nam	Medical Collaboration Center-NCGM/Bach Mai Hospital
7.	Period of the research	September 2013- March 2016
8.	Publications	None
9.	Summary: We started two types of surveys in Viet Nam to evaluate and improve training on health care-associated infection (HCI) control. One is prospective follow-up survey to trace participants in the JICA/NCGM training held in August 2013. Another is retrospective follow-up survey to trace participants in the JICA/NCGM training held before 2012. For retrospective follow-up survey this year we visited Ninh Tuan General Hospital in Ninh Tuan Province that sent a chief doctor of ICT to the training in 2012. We conducted key informant interviews to share information about HCI control with Director of Hospital, Chief Doctor of ICT and Leader of Central Sterile Unit /Infection Control Department. We also conducted site visits including ICU, Infection Control Department and Laboratory etc. Prior to our visit we sent a questionnaire and collected the filled one.	

2. Other activities, topics

International Nursing Practicum for Nursing Students at the National College of Nursing, Japan

We conducted a one-week nursing practicum in Viet Nam as part of the compulsory subject of International Nursing Practicum for fourth-year undergraduate students in collaboration with Hai Duong Medical Technical University (HMTU), Viet Nam.

The International Nursing Practicum is designed to enhance students' abilities to understand the current situation of nursing and health care practice in developing countries, whereby promoting the development of nursing theory with international perspectives to facilitate international health cooperation in nursing. As a prerequisite, students are required to complete the international nursing theory course.

One hundred students were divided into 14 groups, and each group was assigned several presentation topics to work toward the goals of the practicum. Before departing for Hai Duong, Viet Nam, where the practicum took place, students rehearsed their presentation in English in order to improve the quality of presentation and share their knowledge among groups in preparation for the practicum.

On the first day of the practicum, students gave their presentation in front of the faculty members and undergraduate students at HMTU and NCNJ. They then visited several institutions in Hai Duong province, such as provincial hospital, district hospital, special hospital, social welfare institution, and community health center. They also visited Bach Mai Hospital or Viet Duc hospital in Hanoi, both the major referral hospitals.

On the last day of the practicum, each group presented the summary of students' experiences at HMTU. Back in Japan at NCNJ, a poster presentation was held in the entrance hall, which gave students an opportunity to summarize what they had learned through the practicum in both Japan and Viet Nam, as well as to inform other junior students and faculty members of their valuable experiences.

Student evaluation revealed that most students wished to contribute what they had learned to nursing activities in Japan and promotion of international health cooperation.

Asian-African Research Forum on Emerging and Reemerging Infections (AARF) 2014

January 20 - January 22, 2014; Sendai International Center, Sendai; Japan

No.	Title and authors	Presentation
1	**Epidemic genotypes of *Mycobacterium tuberculosis* isolated from Hanoi in Viet Nam** Shinji Maeda, Pham Huu Thuong, Nguyen Van Hung, Nguyen Thi Le Hang, Nobuyuki Kobayashi, Shinsaku Sakurada, Luu Thi Lien, Naoto Keicho	Oral
2	**Circulating adipokines and immune-gene expression levels in patients with multidrug-resistant tuberculosis** Nguyen Thi Bich Yen, Minako Hijikata, Ikumi Matsushita, Nguyen Thi Le Hang, Nguyen Thi Hong, Nguyen Ngoc Lan, Nguyen Huy Dung, Naoto Keicho	Poster
3	**Dual-specificity phosphatase 14 gene polymorphism in Viet Namese patients with pulmonary tuberculosis** Minako Hijikata, Ikumi Matsushita, Nguyen Thi Le Hang, Pham Huu Thuong, Shinsaku Sakurada, Vu Cao Cuong, Luu Thi Lien, Naoto Keicho	Oral
4	**Sublineages of *Mycobacterium tuberculosis* and unfavorable outcomes of anti-tuberculosis treatment** Nguyen Thi Le Hang, Shinji Maeda, Pham Huu Thuong, Nguyen Phuong Hoang, Nguyen Van Hung, Vu Cao Cuong, Minako Hijikata, Shinsaku Sakurada, Luu Thi Lien, Naoto Keicho	Oral
5	**Clinical phenotypes and sublineages of *M. tuberculosis* isolated in Hanoi Viet Nam** Shinji Maeda, Nguyen Thi Le Hang, Pham Huu Thuong, Nguyen Van Hung, Nguyen Phuong Hoang, Vu Cao Cuong, Shinsaku Sakurada, Luu Thi Lien, Naoto Keicho	Oral

Sister Renal Center Program officially approved by International Society of Nephrology (ISN)

Department of Nephrology, NCGM,

* Emerging Center: Department of Nephro-Urology, Bach Mai Hospital (BMH) in Hanoi, Viet Nam
* Supporting Center: Department of Nephrology, NCGM, Shinjuku-ku, Tokyo, Japan

Project Concept

First of all, the ISN Sister Renal Center (SRC) Program (SRCP) helps improve how nephrology is practiced in emerging countries by linking emerging renal centers or units with established centers of excellence in the developed world. Department of Nephrology, NCGM, decided to clinically and technically assist and support Department of Nephro-Urology, BMH for SRCP in 2013. Fortunately, our program was officially approved by ISN in early 2014, and our collaborative project has been going on since then.

BMH is one of the largest, leading hospitals in Northern Viet Nam, focusing on Internal Medicine and medical education, in cooperation with Hanoi Medical University. All of the BMH nephrologists are well educated and have a wide-ranging experience with various projects. On the other hand, the Bureau of International Medical Cooperation, Japan (IMCJ) is a semi-national institute founded in 1986, belonging to NCGM in Japan. It has been functioning as a leading Japanese international cooperation agency in the health sector in association with the Ministry of Health, Labour and Welfare, the Ministry of Foreign Affairs, Japan International Cooperation Agency (JICA) and the World Health Organization (WHO). IMCJ and some of the clinical departments of NCGM have continuously supported BMH to build up a modern medical system and to introduce up-to-date clinical skills. However, there has recently been no collaboration in nephrology between NCGM and BMH. Certainly our SRCP will further raise the capacity of the Department of Nephro-Urology at BMH to instruct many nephrologists working at other local and smaller hospitals in Northern Viet Nam and consequently contribute to the further development at those institutions. In addition to the Department of Nephrology of NCGM, IMCJ which has participated in many international cooperative projects would gladly support the Department of Nephro-Urology at BMH and its nephrologists. Based on SRCP, we believe that both departments from NCGM and BMH can collaboratively start some clinical study in nephrology, resulting in the progress of clinical nephrology and medical care in Viet Nam.

Achievements and Plan

- Two nephrologists of Department of Nephrology, NCGM, and an experienced pathologist were dispatched to Department of Nephro-Urology, BMH, in July, 2014, to give some lectures on the method of evaluating kidney biopsy samples. We also discussed how to promote this SRCP between these 2 hospitals.
- It had been planned that the head of Department of Nephro-Urology, BMH, and two younger medical staff from this department visited Department of Nephrology, NCGM, and learned about the method of skilful hemodialysis and the management or preservative therapy for chronic kidney disease (CKD) practiced here in Japan in January 2015.
- We are planning to further accept other younger medical staff from Department of Nephro-Urology, BMH, in coming March 2015, to dispatch some nephrologists of Department of Nephrology and a medical engineer belonging to NCGM for teaching how to sophisticatedly manage CKD treatment and hemodialysis procedures, and to provide some important textbooks as well as some medical equipment such as automatic needle biopsy system.

III. Reference

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Original article

Low body weight and tenofovir use are risk factors for renal dysfunction in Vietnamese HIV-infected patients. A prospective 18-month observation study

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ABSTRACT

Background: The use of tenofovir has been rapidly increasing in Vietnam. Several studies identified low body weight as a risk factor for tenofovir-induced nephrotoxicity. However, little is known about the impact of tenofovir on renal function in HIV-infected Vietnamese with generally low weight.
Methods: An observational single-center cohort of adult HIV-infected patients on antiretroviral therapy at National Hospital of Tropical Diseases, Hanoi. Patients on tenofovir or with creatinine clearance ≤60 ml/min at baseline were excluded. The incidence of renal dysfunction was compared between patients who switched to tenofovir and those who did not. Renal dysfunction was defined as 25% decline of creatinine clearance from baseline. Time to renal dysfunction was analyzed by the Kaplan–Meier method between the two groups. The Cox hazard model was used to determine risk factors for renal dysfunction in uni- and multivariate analyses.
Results: Of 556 patients enrolled in this study, 403 were non-tenofovir group while 153 were the tenofovir-switched group. Renal dysfunction occurred at a higher rate in the tenofovir-switched group (92.5 per 1000 person-years) than the non-tenofovir group (47.8 per 1000 person-years)($p = 0.023$, Log-rank test). Multivariate analysis confirmed that tenofovir use, low body weight and glucosuria were significant risk factors for renal dysfunction (hazard ratio = 1.980; 95% confidential interval, 1.094–3.582, HR = 1.057; 95%CI, 1.016–1.098, HR = 5.202; 95%CI, 1.245–21.738, respectively).
Conclusions: Tenofovir use, low body weight and glucosuria were significant risk factors for renal dysfunction. We suggest close monitoring of renal function in patients with these risk factors even in resource-limited setting.

Key points

Treatment with TDF and low body weight were significant risk factors for renal dysfunction in Vietnamese HIV-treated patients. Given that the average body weight of Vietnamese is small, close monitoring of renal function in HIV-1-infected patients is important during treatment with TDF.

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1. Introduction

Although renal dysfunction is an important cause of morbidity and mortality in HIV-infected patients [1–7], only limited information is available on renal function in Vietnamese HIV-infected patients. Along with the 2010 WHO guidelines which phased out stavudine and recommended tenofovir (TDF) (URL: httl://whqlibdoc.who.int/publications/2010/9789241599764_eng.pdf), the use of TDF had been increasing in Vietnam in recent years.

TDF-associated nephrotoxicity is well known adverse effect. However, a meta-analysis study that evaluated the safety of TDF concluded that TDF-associated nephrotoxicity can be considered negligible and thus there is no need to restrict TDF use even when regular observation of renal function is not feasible [8]. Other experimental and clinical studies, however; provide a different scenario: one study of rhesus macaques described a dose-dependent nephrotoxic effect for TDF [9] and several studies reported cases of TDF-associated nephrotoxicity in low-body-weight HIV-infected patients [10,11]. Our group also reported that low body weight and use of TDF were significantly associated with chronic kidney dysfunction in Vietnamese HIV-infected patients in a cross-sectional study [12]. Since Vietnamese have a considerably smaller body weight compared with Caucasians, and the use of TDF in Vietnam is increasing throughout the country, the potential risk for TDF-related nephrotoxicity is a concern in Vietnam. This is also true in all countries in the region since the Asian population is, in general, of low body weight. To examine this issue in more detail, we conducted a longitudinal study to evaluate the incidence of renal dysfunction in Vietnamese HIV-infected patients and the risk factors of such morbidity, including use of TDF and low body weight.

2. Patients and methods

2.1. Study design

We performed a prospective observational study of a single-center cohort of Vietnamese HIV-infected patients on antiretroviral therapy (ART) to evaluate the impact of TDF and low body weight on renal function. This cohort was established in 2007 at the National Hospital of Tropical Disease (NHTD) in Hanoi, one of the biggest outpatient clinics for HIV infected-patients in Vietnam. The population of the cohort consists of Vietnamese HIV-infected patients on ART aged more than 17 years referred to NHTD.

To evaluate renal function, serum creatinine had been measured since October 2011, which is the baseline of this study. Entry criteria were patients who were registered in this cohort on October 2011. Patients taking TDF or with serum creatinine clearance (Crcl) of ≤60 ml/min at baseline were excluded. Also excluded from the study were patients whose creatinine was not obtained twice at least. The follow-up period was 18 months (between October 2011 and April 2013). All patients of this cohort received ART at baseline. ART included Zidovudine (AZT)/Lamivudine (3 TC), Stavudine (d4T)/3 TC or TDF/3 TC as nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs) in combination with efavirenz (EFV), Nevirapine (NVP) or ritonavir boosted lopinavir (LPV/r). To estimate the incidence of renal dysfunction in this population, patients were divided into those who switched to TDF and those who did not. Laboratory data, including serum creatinine, were measured twice a year (in April and October) in this cohort. The study was approved by the Human Research Ethics Committee of NHTD. Each patient included in this study provided a written informed consent for the clinical and laboratory data to be used for publication. The study was conducted according to the principles expressed in the Declaration of Helsinki.

2.2. Measurements

Clinical and laboratory data included demographic variables (age, sex and weight), serum creatinine (mg/dl, measured by Jaffe method), CD4 cell count (cell/mm^3, measured by flow cytometry), plasma HIV-RNA (copies/ml, measured by the Roche COBAS TaqMan HIV monitor assay), complete history of ART, use of cotrimoxazole, date of HIV diagnosis, and presence of other comorbidities such as hepatitis B and C virus, diabetes mellitus and AIDS defining diseases. Renal dysfunction was defined as 25% decline in Crcl estimated by the Cockcroft–Gault formula, relative to the baseline.

2.3. Statistical analysis

Baseline characteristics were compared between case patients and control patients by the Student's *t*-test for continuous variables and by either the χ^2 test or Fisher's exact test for categorical variables. The time from baseline to renal dysfunction was analyzed by the Kaplan–Meier method for patients who switched to TDF and those who did not, and the log-rank test was used to determine the statistical significance. Censored cases represented those who died, dropped out, or were referred to other facilities before the end of follow-up period. The Cox proportional hazards regression analysis was used to estimate the impact of TDF use on the incidence of renal dysfunction. The impact of basic demographics, baseline laboratory data, and other medical conditions was also estimated with univariate Cox proportional hazards regression. Variables significantly associated with renal dysfunction in univariate analysis ($p < 0.05$) were entered into multivariate analysis. Statistical significance was defined at two-sided p value < 0.05. We used the hazard ratio (HR) and 95% confidence interval (95%CI) to estimate the association of each variable with renal dysfunction. All analyses were performed in SPSS (version 22.0).

3. Results

At baseline, 793 Vietnamese HIV-infected patients on ART were registered in this study. However, 237 patients were excluded from the study due to existing renal dysfunction at baseline (Crcl < 60 ml/min, $n = 72$), had already been treated with TDF at baseline ($n = 143$), and lack of repeated measurements of Crcl ($n = 22$). Thus, 556 patients who received ART met the study criteria and were included in the study. Of these, 153 patients were switched to TDF during the study period, while 403 patients continued treatment with non-TDF-containing regimen. The criteria for switch to TDF were adverse event caused by ART or induction of treatment for chronic hepatitis B virus infection.

Table 1 compares the baseline demographics and clinical variables of patients of the TDF-switched group and the non-TDF group. The TDF-switched group was significantly more likely to be males, hepatitis B virus S antigen-positive and hepatitis C virus antibody-positive compared to the non-TDF group. The TDF-switched group had marginally significant trend to be older and have diabetes mellitus. Body weight, serum creatinine, CD4 count, HIV RNA viral load, duration of ART, frequency of proteinuria and glucosuria, use of ritonavir boosted lopinavir (LPV/r) and cotrimoxazole, and history of AIDS-defining disease were not significantly different between the two groups. The mean CD4 count was >300/mm^3 and the mean HIV RNA load was <100 copies/ml in both groups.

During the observation period, renal dysfunction, defined as 25% decline in Crcl, was observed in 19 (12.4%) of the TDF-switched group and 27 (6.7%) of the non-TDF group, with an estimated incidence of 92.5 and 47.8 per 1000 person-years, respectively. Fig. 1 depicts the time from the baseline to the development of

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Table 1
Baseline characteristic of Vietnamese patients treated with or without TDF.

Variables	Without TDF	With TDF	*P* value
Number of patients (%)	403 (72.5)	153 (27.5)	
Age, years	35.6 ± 7.0	36.9 ± 6.8	0.064
Women, *n* (%)	167 (41.4)	45 (29.4)	0.009
Body weight	55.7 ± 8.3	56.5 ± 8.2	0.284
Serum creatinine, mg/dl	0.93 ± 0.13	0.93 ± 0.12	0.668
CD4+ cell count, cell/μl	394 ± 197	385 ± 166	0.651
Log 10 HIV-RNA level, copies/ml	1.48 ± 0.55	1.42 ± 0.41	0.190
Proteinuria, *n* (%)	48 (11.9)	21 (13.7)	0.522
Glucosuria, *n* (%)	3 (0.7)	2 (1.3)	0.617
HBVAg (+), *n* (%)	22 (5.5)	29 (18.9)	<0.001
HCVAb (+), *n* (%)	153 (38.0)	69 (45.1)	0.014
Duration of ART, years	1.14 ± 1.35	1.20 ± 1.47	0.650
Use of ritonavir boosted lopinavir, *n* (%)	7 (1.7)	5 (3.3)	0.326
Use of cotrimoxazole drug, *n* (%)	136 (33.7)	45 (29.4)	0.330
Prior AIDS defining disease, *n* (%)	36 (8.9)	12 (7.8)	0.683
Diabetes mellitus (+), *n* (%)	31 (7.7)	19 (12.4)	0.082

Data are expressed as mean ± SD.
ART = Antiretroviral therapy; TDF = tenofovir.

renal dysfunction by Kaplan–Meier method in the two groups. The incidence of renal dysfunction was significantly higher in the TDF-switched group, compared with the non-TDF group ($p = 0.023$, Log-rank test). With regard to the time of switch to TDF, 109 (71.5%) patients of the TDF-switched group switched their nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs) to TDF within 3 months from the baseline and additional 31 (20.0%) switched between 3 and 6 months. Furthermore, of the 19 patients of the TDF-switched group who developed renal dysfunction, 13 (71.2%) switched to TDF within 3 months from the baseline and additional 5 (23.5%) switched to TDF between 3 and 6 months.

Table 2 shows the results of the Cox proportional hazards regression model. Univariate analysis identified body weight per 1 kg-decrement, use of TDF, and glucosuria as factors significantly associated with renal dysfunction. After adjustment by multivariate analysis, body weight per 1 kg-decrement (HR = 1.057; 95%CI, 1.016–1.098; $p = 0.006$), use of TDF (HR = 1.980; 95%CI, 1.094–3.582; $p = 0.024$), and glucosuria (HR = 5.202; 95%CI, 1.245–21.738; $p = 0.024$) were still associated significantly with renal dysfunction.

We also compared the incidence of renal dysfunction in the TDF-switched group according to body weight. Fig. 2 shows the time from baseline to renal dysfunction in patients with body weight of <55 kg, representing the average weight of this study population, and in those with ≥55 kg of the TDF-switched group by Kaplan–Meier method. Patients of the <55 kg group were significantly more likely to develop renal dysfunction [12/66 cases (18.2%), 145.3/1000 person-year] compared to patients of the ≥55 kg group [7/87 cases (8.0%), 57.0/1000 person-year] ($p = 0.040$, Log-rank test).

Table 2
Risk factors for 25% decline in creatinine clearance estimated by uni- and multivariate analyses.

	Univariate analysis			Multivariate analysis		
	HR	95%CI	*P* value	HR	95%CI	*P* value
Age, per year	1.022	0.984–1.061	0.259			
Women	1.484	0.832–2.646	0.181			
Body weight per 1 kg decrease	1.053	1.013–1.094	0.008	1.057	1.016–1.098	0.006
Serum creatinine >1.1 mg/dl	0.397	0.096–1.636	0.201			
CD4+ cell count per cell/μl	1.001	0.999–1.002	0.227			
HIV-RNA level per log 10 copies/ml	0.887	0.446–1.764	0.733			
Proteinuria	0.474	0.147–1.528	0.211			
Glucosuria	5.372	1.301–22.176	0.020	5.202	1.245–21.738	0.024
HBVAg (+)	1.466	0.622–3.458	0.382			
HCVAb (+)	0.949	0.521–1.728	0.864			
Duration of ART per year	1.151	0.970–1.367	0.108			
Use of tenofovir	1.927	1.071–3.465	0.029	1.980	1.094–3.582	0.024
Use of ritonavir boosted lopinavir	2.024	0.491–8.349	0.329			
Use of cotrimoxazole	0.663	0.337–1.305	0.234			
Prior AIDS defining disease	0.043	0.000–4.144	0.177			
Diabetes mellitus (+)	0.952	0.341–2.654	0.925			

HR = hazard ratio; CI = confidence interval; ART = antiretroviral therapy.

The mean serum creatinine was higher in the TDF-switched group compared with the non-TDF group, and the difference in the mean serum creatinine between the two groups increased from 0 mg/dl at baseline, to 0.4 mg/dl at 6 month, 0.5 mg/dl at 12 months and 0.6 mg/dl at 18 months from the baseline.

4. Discussion

In this 18-month prospective study of a single-center cohort, we evaluated the impact of TDF on renal function in Vietnamese HIV-infected patients with low body weight of approximately 55 kg. The Kaplan–Meier curve showed that the cumulative incidence of renal dysfunction was significantly higher among the patients who switched to TDF than among those who did not ($p = 0.023$). Cox proportional hazards regression model identified the use of TDF, low body weight and glucosuria as significant high risk factors for renal dysfunction. In sub-analysis of the TDF-switched group, we confirmed that the cumulative incidence of renal dysfunction was significantly higher in patients with body weight <55 kg compared to those weighing ≥55 kg.

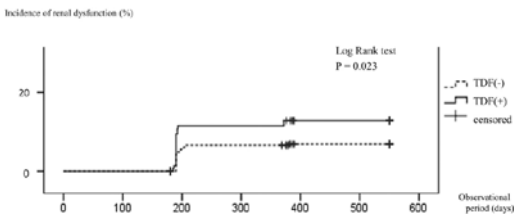

Fig. 1. Kaplan–Meier curve showing the time to renal dysfunction in patients of TDF-switched group and non-TDF-containing groups. Compared to patients of the non-TDF group, those of the TDF-switched group were significantly more likely to develop renal dysfunction ($p = 0.023$, Log-rank test).

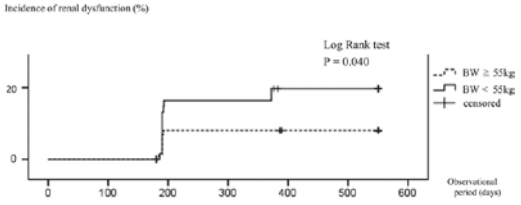

Fig. 2. Kaplan–Meier curve showing the time to renal dysfunction in patients of TDF-switched group classified according to body weight. Compared to patients with body weight ≥55 kg, those weighing <55 kg were significantly likely to develop renal dysfunction ($p = 0.040$, Log-rank test).

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We reported previously that low body weight and TDF use were factors significantly associated with chronic kidney disease in a cross-sectional study of this cohort in Hanoi [12]. The present study confirmed that TDF exposure and low body weight bear a causative relationship to renal dysfunction. We also reported low body weight (<59 kg) as a risk factor for renal dysfunction in Japanese patients treated with TDF [10], whereas high body weight of >67 kg was not the risk, similar to the body weight of the patients reported by Cooper et al. [8]. In light of the fact that the average body weight of the patients in this cohort was 55 kg, which is around 30 kg lighter than that of average American males (88 kg) (URL:http://www.cdc.gov/nchs/data/nhsr/nhsr010.pdf), the impact of these risk factors on renal function remain unknown in patients with low body weight in the long-run, thus, observational studies will need to be continued for a longer term.

In addition to low body weight, the presence of glucosuria at baseline was identified as a risk factor for renal dysfunction. This result is consistent with the most recent WHO guidelines which suggest urinary glucose as one of the cost-effective screening test for serious TDF-induced kidney injury (URL: http://apps.who.int/iris/bitstream/10665/85321/1/9789241505727_eng.pdf). Since the number of patients with glucosuria was small in this study (about 1% of total population), and glucosuria was not followed until the end of the observation period, further evaluation of this factor is necessary.

Other risk factors for renal dysfunction described in previous studies, such as cotrimoxazole, LPV/r, hepatitis C virus co-infection and diabetes mellitus [13–16] were not identified as risk factors in this study. This discrepancy could be explained by the fact that patients who could be affected by these factors were already excluded according to the study design, which excluded patients with renal dysfunction. With regard to the use of LPV/r, which is known as a risk for renal dysfunction [14,17], especially in cases of co-use with TDF, a number of patients with LPV/r were excluded from the study since most of the patients with LPV/r were co-treated with TDF at baseline. Thus, the impact of co-use of LPV/r and TDF on renal function could be underestimated in this study. Given that LPV/r is used as a salvage regimen and often administered with TDF in Vietnam, long-term monitoring of renal function is required in patients treated with both LPV/r and TDF.

The present study has several limitations. First, data on hypertension, which is a risk factor for renal dysfunction, were not available in this study. Although the average age of patients in this study was around 36 years and the prevalence of hypertension may not high, measurement of blood pressure could lead to better management of renal dysfunction and hypertension should be evaluated for potential risk. Regarding diabetes mellitus as well, the degree of diabetes mellitus was not checked in detail. However, severe patients such as insulin dependence were not in this study, thus, the lack of data could be limited. Second, the observation period of 18 months is relatively short to evaluate long-term adverse event for renal function as mentioned above. Some studies advocated stabilization of decline in eGFR later after the first 6 months of TDF exposure [18] and reversibility of eGFR decline after cessation of TDF therapy [19], while several studies argued incomplete reversibility of eGFR decline following TDF exposure [20–22]. In this study, most of the patients who developed the decline in Crcl continued the same ART regimen because of their moderate and/or stabilized renal dysfunction. However, the observational period of the present study is relatively short compared to other studies, thus, whether or not the stabilization and reversibility will be observed in this cohort of averagely small body weight should be evaluated in the longer period.

Third, the timing of switch to TDF and total duration of ART were not unified in the present study, since the study was an observational cohort in which patients were already on ART at enrollment. The reasons for switch to TDF were mainly related to adverse events caused by d4T and AZT or treatment for HBV infection, thus the timing of switch to TDF was not strictly controlled. However, more than 70% and 90% of the patients were switched to TDF within 3 and 6 months from baseline, respectively, thus influence of this limitation on the result of this study could be restricted.

Despite concern on nephrotoxicity, TDF remains an important drug with enough anti-HIV potency and less mitochondrial toxicity among NRTIs. In order to use it safely in the long term, serum creatinine should be monitored in patients with aforementioned risk factors even in resource-limited situations. Further longitudinal studies are required to determine the impact of TDF, low body weight and glucosuria on renal function in Vietnamese and other Asian people with low body weight.

Conflict of interest

S.O. has received honoraria and research grants from MSD K.K., Abbott Japan, Co., Janssen Pharmaceutical K.K., Pfizer, Co., and Roche Diagnostics K.K.; received honoraria from Astellas Pharmaceutical K.K., Bristol-Myers K.K., Daiichisankyo, Co., Dainippon Sumitomo Pharma, Co., GlaxoSmithKline, K.K., Taisho Toyama Pharmaceutical, Co., Torii Pharmaceutical, Co., and ViiV Healthcare. H.G. has received honoraria from MSD K.K., Abbott Japan, Co., Janssen Pharmaceutical K.K., Torii Pharmaceutical, Co., and ViiV Healthcare, Co. All other authors declare no conflict of interest.

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Low Prevalence of Transmitted Drug Resistance of HIV-1 During 2008–2012 Antiretroviral Therapy Scaling up in Southern Vietnam

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Background: The recent expansion of antiretroviral therapy (ART) program in resource-limited setting has raised concern about possible transmission of drug resistance (TDR). We assessed the prevalence of TDR over a 5-year period among treatment-naive individuals in Southern Vietnam during rapid ART scale-up.

Methods: Drug resistance mutations among antiretroviral-naive HIV-1–infected patients in Ho Chi Minh City were evaluated prospectively from 2008 to 2012 by HIV-1 pol gene sequencing. TDR was defined according to the World Health Organization list for surveillance of transmitted HIV-1 drug resistance in 2009.

Results: Pol sequence was obtained in 1389 individuals (median age: 30 years, males: 52.3%). Risks of HIV-1 infection included heterosexual contact in 60.7%, injection drug use in 22.4% and both 5.2%. The majority was infected with CRF01_AE (97%), whereas 19 were infected with subtype B. Over the 5-year study period, TDR was detected in 58 individuals (4.18%): 28 (2.02%) against nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs), 19 (1.37%) against nonnucleoside reverse transcriptase inhibitors (NNRTIs), and 15 (1.08%) against protease inhibitors (PIs), including 4 (0.29%) against both NRTIs and NNRTIs. The most common TDR was K103N (0.5%) for NNRTI. The annual prevalence of TDR remained low to moderate (2008: 2.4%; 2009: 5.2%; 2010: 5.48%; 2011: 2.72%; 2012: 5.36%), and there was no clear trend over time.

Conclusions: There was no increase in TDR prevalence in Southern Vietnam during and after the 2008–2012 rapid scale up of ART.

Key Words: HIV, transmitted drug resistance, Vietnam

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INTRODUCTION

The recent roll-out campaigns in resource-limited settings to scale up antiretroviral therapy (ART) seem to have improved the morbidity and mortality of HIV-infected individuals. In Vietnam, where the HIV epidemic affected 249,660 individuals including 52,325 AIDS-related deaths up to the end of 2011, a national effort to facilitate ART supply has been implemented, and the ART coverage rate has rapidly increased from 18.1% in 2006 to 53% in 2011, saving 18,110 lives from AIDS-related deaths between 2000 and 2009.[1]

The expansion of ART program, however, has been accompanied by concerns on HIV drug resistance and risk of subsequent transmission of drug resistance (TDR) in new cases of HIV infection.[2] The WHO recommends surveillance of TDR where ART is being scaled up[3,4] and the Vietnam Authority of HIV/AIDS Control issued in 2008 a 5-year plan to assess and prevent HIV drug resistance. Because the large part of HIV epidemic in Vietnam has been driven by intravenous drug users (IDUs),[1,5] it is theoretically possible that the transmission of drug-resistant HIV spreads fast by sharing contaminated needles. The recent increase in HIV transmission by sexual intercourse in Vietnam also makes the TDR problem more difficult to control.[5] In addition, the pattern of antiretroviral drug use has been changing according to the global policy on ART recommendations or increased availability of second-line ART.[6–9] It is therefore important to monitor the prevalence of TDR and its pattern in Vietnam on a regular basis. Previous surveys and studies demonstrated low-to-moderate prevalence of TDR in Vietnam.[10–17] However, those studies were conducted using a cross-sectional setting or included monitoring for only a short period of time. To the best of our knowledge, there are no data on long-term monitoring of the prevalence of TDR in Vietnam.

This study was designed to assess the prevalence of TDR over a 5-year period in HIV-infected treatment-naive

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individuals from Southern Vietnam during the 2008–2012 rapid ART scale-up.

METHODS

Study Population

Antiretroviral-naive individuals who visited the Hospital for Tropical Diseases in Ho Chi Minh City, Vietnam, were enrolled in the study from 2008 until 2012. The enrollment of consecutive antiretroviral-naive patients started in October and ended when 300 enrollments had been achieved. In 2009 and 2012, the enrollment was stopped at 250 and 270, respectively, for the operational reasons. After securing written informed consent, plasma samples were collected and stored at −80°C. At the end of the year's sampling, the frozen plasma samples were shipped to the National Center for Global Health and Medicine (NCGM) in Tokyo, Japan, for genotypic resistance testing. Patients with history of exposure to any antiretroviral drug, including mono or dual therapy were excluded. The study protocol was approved by the institutional ethical review boards of both Hospital for Tropical Diseases in Vietnam and NCGM in Japan (NCGM#360).

Genotypic HIV-1 Resistance Testing and Subtype Determinations

Drug resistance genotyping was performed using in-house protocols at NCGM. Briefly, total RNA was extracted from plasma with a High Pure Viral RNA kit (Boehringer Mannheim, Mannheim, Germany), followed by reverse transcription–polymerase chain reaction (PCR) with a One Step RNA PCR kit (TaKaRa Shuzo, Otsu, Japan). Nested PCR was subsequently conducted with a Prime STAR Max Premix kit (TaKaRa Shuzo, Otsu, Japan) to amplify the pol-reverse transcriptase (RT) and protease (PR) region. The PCR products were purified with QIAquick PCR Purification Kit (Qiagen, Valencia, CA) and subjected to direct sequencing with an ABI PRISM 3730 automated DNA sequencer (Applied Biosystems, Foster City, CA). Amino acid sequences were deduced with the Genetyx-Win program version 11.0 (Software Development, Tokyo). The subtypes of HIV-1 were determined by using RT gene with "Genotyping/NCBI" tool using BLAST algorithm (http://www.ncbi.nlm.nih.gov/projects/genotyping/formpage.cgi). Drug resistance mutations were identified from the list for surveillance of transmitted drug resistance mutations.[18] All sequences obtained from the study have been deposited in the DNA Data Bank of Japan database (accession no: AB894875 to AB896651).

Statistical Analysis

Differences between 2 groups were tested for statistical significance by using χ^2 test for categorical data and the Mann–Whitney test for continuous variables. Logistic regression model was used to identify the factors associated with infection by TDR. Differences were considered significant if the P value was less than 0.05. Statistical analyses were performed using IBM SPSS Statistics software version 21J (IBM Japan, Inc, Tokyo, Japan).

RESULTS

Characteristics of Study Population

The study enrolled 1426 individuals but 20 were later found to be ineligible after providing written informed consents (previous ART exposure, n = 17, insufficient blood withdrawn, n = 2 had, negative for HIV infection, n = 1). The remaining 1406 participants were assigned to the drug resistance test. The characteristics of these subjects are summarized in Table 1. Approximately 63% of the study participants were men, and the latter were older than females (31 years vs 29 years; $P < 0.001$). The most frequently reported HIV transmission route was heterosexual contact (65.9%), followed by injection drug use (IDU) (29.6%). Very few (0.1%) declared homosexual contact as a risk for HIV infection. The majority of patients with IDU were men, and the percentage of IDUs was greater in men than that in women (men: 42%; women: 3.4%; $P < 0.001$). The proportion of IDUs had decreased over time (35% in 2008, 17.6% in 2012) and the prevalence of hepatitis C infection, which reflects possible multiple needle sharing, had decreased simultaneously. These changes in the study population reflect preponderance of HIV epidemic in male IDUs in the early phase and recent expansion to the general population in Vietnam.[1]

Prevalence of Transmitted Drug Resistance Mutations

Among the 1406 individuals who underwent HIV drug resistance genotyping, we obtained the complete sequences of both PR and RT in 1389 individuals. The majority were infected with CRF01_AE (98%), whereas 17 were infected with subtype B. Over the 5-year study period, drug resistance mutations were detected in 58 individuals (4.18%): 28 (2.02%) against nucleos(t)ide reverse transcriptase inhibitors (NRTIs), 19 (1.37%) against nonnucleoside reverse transcriptase inhibitors (NNRTIs), and 15 (1.08%) against protease inhibitors (PIs), including 4 against both NRTIs and NNRTIs. Table 2 summarizes the prevalence of the specific drug resistance mutations. The annual prevalence of TDR was persistently low during the study period, ranging from 2.40% to 5.48%, and no clear trend was noted over time. In thymidine analog mutations (TAMs), mutations at codon 215 were the most frequent (0.36%) followed by K219Q (0.22%). In other NRTI-related mutations, V75M and mutations at codon 74 and 184 were relatively frequent, of which V75M was reported previously as frequent d4T-resistance-related mutation among CRF01_AE.[19] We did not identify mutations related to Q151M complex or insertions at codon 69. The most common NNRTI mutation was K103N (0.5%), followed by Y181C (0.43%), G190A and E (0.36%), and mutations at codon 188 (0.22%). The most common PI-associated mutations was M46L (0.43%) and M46I (0.29%) but both were considered polymorphisms.[20,21] All other PI-associated mutations were rare; only 1 among 1389 sample (0.07%) harbored each mutation. Of those, D30N, L76V, and L90M were major mutations, whereas F53Y was not major mutation[20,21] and not clinically significant when it occurred alone without any other PI mutations.

TABLE 1. Patients' Characteristics

		Year of Sampling				
	Total	2008	2009	2010	2011	2012
Patients, n	1406	298	250	294	297	267
Male gender, n (%)	881 (62.7)	213 (71.5)	150 (60)	184 (62.6)	154 (51.9)	180 (67.4)
Age, median (range)	30 (16–66)	29 (16–58)	29 (20–60)	30 (17–55)	31 (19–66)	33 (18–65)
Living in HCMC, n (%)	735 (52.3)	163 (54.7)	132 (52.8)	148 (50.3)	150 (50.5)	142 (53.2)
Time since HIV diagnosis, n (%)						
<6 mo	975 (69.3	224 (75.2)	181 (72.4)	233 (79.3)	138 (46.5)	199 (74.5)
≥6 mo	431 (30.7)	74 (24.8)	69 (27.6)	61 (20.7)	159 (53.5)	68 (25.5)
Risk of HIV transmission, n (%)						
Heterosexual contact, alone	854 (60.7)	148 (49.7)	143 (57.2)	149 (50.7)	210 (70.7)	204 (76.4)
IDU, alone	315 (22.4)	73 (24.5)	68 (27.2)	90 (30.6)	44 (14.8)	40 (15.0)
Heterosexual and IDU	73 (5.2)	29 (9.7)	5 (2)	3 (1)	29 (9.8)	7 (2.6)
Homosexual contact	2 (0.1)	2 (0.7)	0	0	0	0
Other/unknown	162 (11.5)	46 (15.4)	34 (13.6)	52 (17.7)	14 (4.7)	16 (6.0)
HIV-1 subtype, n (%)						
CRF01_AE	1378 (98.0)	295 (99.0)	246 (98.4)	289 (98.3)	289 (97.3)	255 (95.5)
Subtype B	19 (1.5)	1 (0.7)	4 (1.6)	2 (1)	6 (2)	6 (2.2)
Other/unclassified	9 (0.8)	0	0	2 (0.7)	1 (0.6)	6 (2.2)
HBs antigen positive, n (%)	217 (15.4)	42 (14.1)	43 (17.2)	49 (16.7)	47 (15.8)	36 (13.5)
Anti-HCV antibody positive, n (%)	557 (39.6)	148 (49.7)	106 (42.4)	117 (39.8)	105 (35.4)	81 (30.3)
CD4 cell count, cells/μL, median (range)	110 (1–1322)	70 (1–1042)	115 (1–753)	95 (1–1048)	253 (2–1322)	47 (1–1211)
Plasma HIV-1 RNA levels, log copies/mL, median (range)	5.01 (1.59–6.90)	4.81 (1.69–5.70)	4.38 (1.69–5.70)	5.23 (1.59–6.61)	5.02 (2.31–6.90)	5.38 (1.60–6.83)

HCMC, Ho Chi Minh City; CRF01_AE, circulating recombinant form01_AE; HBs antigen, hepatitis B virus surface antigen; anti-HCV antibody, anti-hepatitis C virus antibody.

The presence of TDR did not correlate with any specific demographic factor, risk group, or year of study enrollment, although the odds ratio of acquiring TDR was relatively low in heterosexual individuals (Table 3). Annual trends of TDR prevalence in particular HIV risk categories are shown in Table 4. TDR prevalence in heterosexual contact alone, IDU alone, and IDU plus heterosexual contact were 3.33%, 5.41%, and 2.78% respectively, which were not statistically different. Although no significant annual trend was noted over the study period among them, the TDR prevalence in the HIV risk group of IDU alone were higher than the WHO first threshold 5% in the year 2009, 2010, and 2012 (4.10% in 2008, 5.88% in 2009, 6.67% in 2010, 2.27% in 2011%, and 7.69% in 2012). Phylogenetic tree analysis showed no clustering of sequences from the study participants with TDR. Details of the 4 individuals with TDR in more than 1 group of antiretrovirals are listed in Table 5. One individual had very extensive resistance: M41L, M184V, T215Y in NRTI-associated mutations, and Y181C and G190A in NNRTI-associated mutations. Overall, persistently low prevalence of TDR during the last 5 years of ART expansion was noted. However, individuals with multiple-drug resistances were identified during ART expansion. This finding highlights the importance of TDR and undermines the efficacy of currently scaled up ART regimens.

DISCUSSION

In this study, we traced the prevalence of TDR over a relatively long period of time (from 2008 to 2012) in treatment-naive individuals in Southern Vietnam during rapid ART scaling up program. Our result of 4.18% of overall TDR prevalence was similar to those described previously in Vietnam.[10–17] However, the study covered longer period of time and demonstrated the stability of TDR prevalence over this period. In comparison, all the other previous surveillance studies conducted in Vietnam were shorter in duration. Primary HIV drug resistance is one of the main concerns in any ART program because it can compromise the clinical outcome of ART, especially in countries with limited ART options. Our data of persistently low prevalence of TDR in Southern Vietnam possibly reflect the success of the recent ART scale-up program in this country.

The TDR rate in our study, however, ranged from 2.4% to 5.5%, reaching the threshold of low prevalence according to the WHO definition (<5%) in 2009, 2010, and 2012.[4] Considering lower viral replication fitness of strains harboring drug resistance mutations than that of wild-type strain, the rate of pretreatment resistance in chronic HIV infection could underestimate the real drug resistance transmission with time since HIV infection. In particular, the low-level prevalence of M184V[22] despite widespread use of lamivudine, which is sometimes used for treatment of hepatitis B virus infection, could be related to the lower viral fitness. Of note, the percentage of individuals diagnosed as HIV positive more than 6 months before study enrollment was higher in 2011 (53.5%) than that in other study periods, and the TDR prevalence in 2011 was lower (2.72%) than that in 2009, 2010, and 2012. Most cases had chronic HIV infection at the time of HIV

TABLE 2. Prevalence of Transmitted Drug Resistance Mutations

	Total	2008	2009	2010	2011	2012
Study population (n)	1389	292	250	292	294	261
Any TDR [n (%)]	58 (4.18)	7 (2.40)	13 (5.20)	16 (5.48)	8 (2.72)	14 (5.36)
RT in total [n (%)]	43 (3.10)	7 (2.40)	9 (3.60)	14 (4.79)	4 (1.36)	10 (3.83)
NRTI [n (%)]						
Any	28 (2.02)	3 (1.03)	6 (2.40)	11 (3.76)	3 (1.02)	5 (1.92)
Thymidine analog mutations						
M41L	2 (0.14)			1	1	
D67N	1 (0.07)		1			
D67E	1 (0.07)			1		
K70E	1 (0.07)			1		
T215Y	1 (0.07)				1	
T215I	1 (0.07)		1			
T215S	1 (0.07)				1	
T215D	2 (0.14)		2			
K219Q	3 (0.22)		1	2		
Others						
K65R	2 (0.14)			2		
L74V	1 (0.07)	1				
L74I	4 (0.29)	1		2		1
V75M	6 (0.43)	1		2		3
M184V	3 (0.22)		1		2	
M184I	2 (0.14)			1		1
NNRTI [n (%)]						
Any	19 (1.37)	5 (1.71)	3 (1.20)	4 (1.37)	3 (1.02)	4 (1.53)
K101E	4 (0.29)	1	2	1		
K103N	7 (0.50)	1	1	1		4
Y181C	6 (0.43)	1		2	1	2
Y188L	1 (0.07)				1	
Y188H	1 (0.07)			1		
Y188C	1 (0.07)			1		
G190A	4 (0.29)	2		1	1	
G190E	1 (0.07)				1	
PI [n (%)]						
Any	15 (1.08)	0	4 (1.60)	2 (0.68)	4 (1.36)	5 (1.92)
D30N	1 (0.07)				1	
M46I	4 (0.29)		2			2
M46L	6 (0.43)		1		3	2
M46I/L	1 (0.07)			1		
F53Y	1 (0.07)			1		
L76V	1 (0.07)					1
L90M	1 (0.07)		1			

diagnosis, and the exact latency from infection to diagnosis or to study enrollment was unavailable. Thus, the longer duration from diagnosis to study participation allows more frequent reversion from TDR into wild-type virus. This should be taken into account in the interpretation of the results of the study.

Although our study participants did not represent the national HIV-infected population in Vietnam but were rather HIV-infected individuals living in or near Ho Chi Minh City (HCMC), their age, sex, and the distribution of HIV risks were almost comparable with the national HIV-infected population in Vietnam. Notably, HCMC accounts for approximately 50% of the entire population receiving ART in Vietna,[12] and ART had been widely accessible in HCMC since the early phase of ART scale-up or even before ART scale-up at private clinics. Since previous studies had predicted increased TDR rates after 5–8 years of ART scale-up,[2] HIV-infected individuals in HCMC are considered to be at higher risk of TDR compared with those in other areas of Vietnam. In addition, a previous study conducted in HCMC showed that 73% of patients on ART reported having injected drugs,[1] and the sentinel surveillance in 2009 showed that HCMC had high HIV prevalence among IDUs (46%).[1] Since IDU is considered a risk factor for poor adherence and emergence of drug resistance,[23,24] patients in HCMC are considered the key population for TDR monitoring. Although no statistical relationship was

TABLE 3. Relation Between Demographic and Clinical Factors and the Presence of Transmitted Drug Resistance

	With TDR (n = 58)	Without TDR (n = 1331)	Odds Ratio*	95% CI	*P* Value
Male gender, n (%)	42	831	1.58	0.88 to 2.53	0.13
Age (yrs), n (%)					
<30	24	538	1.00		
30–39	22	557	0.98	0.54 to 1.78	0.95
≥40	12	178	1.67	0.82 to 3.42	0.16
Time since HIV diagnosis, n (%)					
<6 mo	46	923	1.00		
≥6 mo	12	401	0.60	0.31 to 1.15	0.12
Unknown	0	7			
Year of HIV diagnosis					
Before 2008	2	132	1.00		
2008	6	223	1.78	0.35 to 8.93	0.49
2009	13	301	2.85	0.63 to 12.8	0.17
2010	17	293	3.83	0.87 to 16.8	0.08
2011	8	149	3.54	0.74 to 17.0	0.11
2012	12	226	3.50	0.77 to 15.9	0.10
Unknown	0	7			
Year of study enrollment, n (%)					
2008	7	285	1.00		
2009	13	237	2.23	0.88 to 5.69	0.09
2010	16	276	2.36	0.96 to 5.83	0.06
2011	8	286	1.14	0.40 to 3.18	0.80
2012	14	247	2.31	0.92 to 5.81	0.08
Risk of HIV transmission, n (%)					
Heterosexual contact	30	883	0.60	0.33 to 1.09	0.05
Injection drug use	19	367	1.49	0.82 to 2.69	0.19
Other	1	20	1.19	0.16 to 9.07	0.86
Unknown	10	131			
HBs antigen positive, n (%)	12	205	1.43	0.74 to 2.74	0.28
HCV antibody positive, n (%)	19	533	0.72	0.41 to 1.27	0.26
CD4 cell count, cells/μl					
≥100	24	686	1.00		
<100	34	642	1.51	0.89 to 2.58	0.14
Unavailable	0	3			

*Logistic regression model was used for calculating odds ratio.
CI, confidence interval.

found in our study between TDR and various risk factors, the odds ratio was lowest for heterosexual contact, with a marginal *P* value of 0.05, which indirectly suggests that other risk groups, such as IDU or men who have sex with men, is at higher risk of TDR. Meanwhile, the proportion of IDUs in our study had decreased during the 5 years along with the nationwide shift from the concentrated HIV epidemic in male IDUs to the general population. Although we failed to find the statistical impact of HIV risk group on TDR prevalence, TDR prevalence among IDU were

TABLE 4. Prevalence of Transmitted Drug Resistance Mutations in Specific HIV Risk Categories

	Total	2008	2009	2010	2011	2012
Total TDR rate [% (n/total)]	4.18 (58/1389)	2.40 (7/292)	5.20 (13/250)	5.48 (16/292)	2.72 (8/294)	5.36 (14/261)
TDR rate in HIV risk categories [% (n/total)]						
Heterosexual contact alone	3.33 (28/840)	1.40 (2/143)	4.90 (7/143)	3.40 (5/147)	1.92 (4/208)	5.02 (10/199)
IDU alone	5.41 (17/314)	4.10 (3/73)	5.88 (4/68)	6.67 (6/90)	2.27 (1/44)	7.69 (3/39)
IDU plus heterosexual	2.78 (2/72)	3.45 (1/29)	0 (0/5)	0 (0/3)	3.57 (1/28)	0 (0/7)
Homosexual contact alone	0 (0/2)	0 (0/2)	- (0/0)	- (0/0)	- (0/0)	- (0/0)
Other	0 (0/20)	0 (0/13)	0 (0/3)	0 (0/1)	0 (0/3)	- (0/0)
Unknown	7.80 (11/141)	3.13 (1/32)	6.45 (2/31)	9.80 (5/51)	18.2 (2/11)	6.25 (1/16)

TABLE 5. Characteristics of 4 Patients With Drug Resistance Mutations Against Multiple Class Antiretrovirals

Patient ID	Year of HIV Diagnosis	Year of Study Participant	Sex	Risk of HIV Infection	CD4 Count (Cells/μL)	HIV-RNA (Log Copies/mL)	HBs Antigen	HCV Antibody	Resistance Mutations	
									NRTI	NNRTI
08HT0059	2003	2008	M	Heterosexual	10	4.11	Negative	Negative	L74V	V106I, G190A
10HT0136	2010	2010	F	Unknown	283	4.60	Negative	Negative	D67E	Y188C
11HT0201	2011	2011	F	Heterosexual	272	5.98	Positive	Negative	M41L, M184V, T215Y	Y181C, G190A
11HT0299	2011	2011	M	Unknown	147	5.83	Negative	Negative	M184V	V106I, V179D, Y188L

relatively higher, which was above 5% in 2009, 2010, and 2012 and had changed along with the overall TDR prevalence. These findings support that IDU is still important as a TDR risk factor in this population. In this regard, however, our study enrolled 141 patients who were free of possible HIV risk or refused to provide information on their risky behavior. Because their TDR prevalence was high over the study period, their concealment of IDU experience could influence the analysis. Although our study was conducted only in urban area, our findings in individuals at most risk of TDR are useful for the assessment of the situation in the near future of the entire HIV population in Vietnam, including rural area where ART has been rapidly distributed in recent years.

With respect to the drug class, the TDR prevalence was 2.02% for NRTI, 1.37% for NNRTI, and 1.08% for PI. Compared with the TDR rate for CRF01_AE strain in the TDR lists for surveillance[18] (2.9% for NRTI, 0.5% for NNRTI, and 1.5% for PI), the TDR prevalence of NNRTI-related mutations was higher for the entire study period and considered to have increased with ART scale-up. The Vietnamese national guideline for ART recommended nevirapine as one of the first-line regimen in 2005 and either nevirapine and efavirenz since 2009,[6–8] and generally NNRTI-base regimens have low genetic barriers for development of drug resistance. This background provides reasonable explanation of frequent detection of NNRTI-related mutations like in other resource-limited countries. However, TAMs and M184V or I were predominantly seen in NRTI-related mutations, which have clinically significant impact on treatment outcome. Even after changing the first-line NRTI in the national ART guideline from zidovudine (AZT) or stavudine (d4T) into tenofovir (TDF) in 2010, AZT or d4T were still extensively used in Vietnam over the study period. In Western Europe, a decline in the prevalence of TAMs is being observed in treatment-experienced cohort as a consequence of changing prescription patterns and prompt management of treatment failure.[25,26] Therefore, the TDR patterns in Vietnam could be changed with future increase in TDF use and decrease in AZT or d4T use. We should note that 4 individuals in our study had TDR in multiple drug classes, including 1 who had very extensive resistance: M41L, M184V, T215Y in NRTI and Y181C and G190A in NNRTI, which strongly compromise the efficacy of the first-line regimens in Vietnam and could threaten the nationwide ART scale-up program if it spreads. There are multiple factors that influence the prevalence of individual resistance mutations in primary HIV drug resistance but treatment-experienced persons with resistance might be the main source of such multiple-class TDR. Although continuous TDR surveillance is important to catch TDR expansion, efforts to enhance early diagnosis of treatment failure with improvement in availability of tests for plasma viral load and drug resistance in individuals on treatment, should be encouraged to prevent transmission of drug-resistant HIV.

In conclusion, TDR prevalence in Southern Vietnam remained low during the rapid scale-up of ART in 2008–2012. No demographic factor was statistically related to TDR detection, and the patterns of detected TDRs were similar to those described in previous reports. Although the average TDR prevalence was low, moderate prevalence was noted in part of the study period, and multiple-class TDR was detected in some patients. Because ART will continue to be scaled up, the TDR rate can rise in the future. Our results highlight the importance of TDR surveillance over a long period of time to provide proper assessment of the ART scale-up program.

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EPIDEMIOLOGY

Mycobacterium tuberculosis strains spreading in Hanoi, Vietnam: Beijing sublineages, genotypes, drug susceptibility patterns, and host factors

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SUMMARY

Beijing genotype strains are divided into two major sublineages, ancient (atypical) and modern (typical) types, but their phenotypic variations remain largely unknown. *Mycobacterium tuberculosis* (MTB) isolates from Hanoi, Vietnam, were analyzed by single-nucleotide polymorphisms and spoligotyping. Patient information and drug susceptibility patterns were obtained. Genetic clustering was assessed by variable number of tandem repeat (VNTR) locus sets. Multivariate analysis was also performed to investigate factors possibly associated with these sublineages. Of the 465 strains tested, 175 (37.6%) belonged to the ancient Beijing sublineage and 97 (20.9%) were of the modern Beijing sublineage. Patients with the Beijing genotype were significantly younger and more undernourished than those with non-Beijing genotype. The proportion of clustered strains calculated from 15 locus-optimized mycobacterial interspersed repetitive units [optimized-(MIRU)15]-, optimized-MIRU24-, optimized-MIRU28-, Japan Anti-Tuberculosis Association (JATA)15-, and JATA18-VNTRs were 55.7%, 49.2%, 33.8%, 44.5%, and 32.0%, respectively. Ancient and modern Beijing genotype strains were more frequently clustered than non-Beijing genotype strains, even when using VNTR sets with high discriminatory power. Isoniazid and streptomycin resistance tended to be more frequently observed in ancient Beijing strains than in modern Beijing strains and others. Our findings may provide insight into area-dependent differences in Beijing family strain characteristics.

1. Introduction

Tuberculosis remains a major public health problem, with an estimated 8.8 million cases and almost 1.4 million deaths occurring annually worldwide [1]. The global population structure of the pathogen *Mycobacterium tuberculosis* (MTB) is currently defined by seven major lineages, of which the Beijing genotype family belongs to the East-Asian lineage [2]. This genotype represents more than 50% of strains in East Asian areas [3].

Studies report that Beijing genotype strains are becoming widespread, even outside Asia. It is possible that this occurs through the exploitation of an imperfect host immune system or it

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may be associated with antibiotic resistance, including multidrug-resistant TB [4,5]. However, the results of these studies are not always consistent, and phenotypic variations in major subtypes within the Beijing genotype remain largely unknown [5]. *In vitro* studies [6] and evidence from studies using animal models [7] have shown that the hypervirulence displayed by Beijing genotype strains is not common to all members of the Beijing family but is restricted to some subsets of the strains.

Beijing genotype strains are divided into two major sublineages, ancient (atypical) and modern (typical), according to the absence or presence of an IS*6110* insertion, respectively, in a particular chromosomal position designated as the NTF region [8]. Modern Beijing genotype strains are prevalent around the northern area of mainland China and extend to the former Soviet Union and South Africa [9], while ancient Beijing genotype strains are predominant in Japan and Korea [10–12].

Vietnam is a Southeast Asian country stretching over 1800 km from north to south. To the south of Vietnam, the Beijing genotype strains seem to be predominant in hospital settings, ranging from 53% [13] to 82% [14], but have been shown by population-based studies to be less predominant in rural areas (35.6% [15]). So far, except for one study conducted on the proportion of sublineages among the Beijing family in the south of Vietnam [16] from 1998 to 1999, there have been no comprehensive studies focused on the phenotypic variations of the ancient and modern Beijing genotypes in this country. Thus, we investigated the prevalence and characteristics of MTB lineages and sublineages in the north of Vietnam (Hanoi, the capital city) circulating among patients newly diagnosed with pulmonary TB and identified factors possibly associated with the Beijing genotypes. To confirm which sublineage of the Beijing genotype strains is predominant, we also tested another set of MTB samples isolated in Hanoi.

It is also important to know the clustering information of the MTB isolates in this area as it may indicate recent transmission events [17,18]. To investigate the genetic clustering of the MTB lineages and sublineages, we tested different locus sets of the variable number of tandem repeat (VNTR) genotyping system: These included two international standard typing systems; the 15 and 24 locus sets (optimized-mycobacterial interspersed repetitive units [MIRU] 15- and -MIRU24-VNTR) and three others recommended for the Beijing genotype strains; a new set (optimized-MIRU28-VNTR) consisting of 24 loci of the optimized-MIRU24 plus four additional loci [VNTR-1982 and three hypervariable (HV) loci (VNTR-3232, -3820, and -4120)], which was recently recommended by the Pasteur Institute in France [19], the Japan Anti-Tuberculosis Association (JATA)15-VNTR set consisting of JATA12-VNTR [20] plus three loci (ETR-A, VNTR-1982, and -2163a), and the JATA18-VNTR set consisting of JATA15 plus the three aforementioned HV loci, of which the JATA12 or 15 system has been integrated into the TB control system nationwide and is currently used in Japan, where Beijing strains are predominant [21]. We also assessed the performance of these systems.

2. Materials and methods

2.1. Study sites, recruitment of patients, and ethics statement

MTB isolates were collected as a part of a cohort study [22]. Written informed consent was obtained from each participant. In the case of minors, their parents provided written informed consent. This study was approved by the Ethical Committees of the Ministry of Health, Vietnam, National Center for Global Health and Medicine, and the Research Institute of Tuberculosis, Japan Anti-Tuberculosis Association, Japan.

From July 2007 to March 2009, seven districts of Hanoi city in Vietnam were enrolled as a study area, and approximately 70% of patients diagnosed with new smear-positive pulmonary TB in the districts agreed to participate in this study. All the participants were Vietnamese. They received the standard 8-month regimen of 2S(E)HRZ/6HE, which was commonly administered during the study period in Vietnam. In the current study, only one isolate per patient at the time of diagnosis was used for analysis. We also tested another set of DNA samples that were consecutively collected using mycobacteria growth indicator tubes (MGIT) in the Hanoi Lung Hospital in 2011.

2.2. Identification of MTB, drug susceptibility testing, and molecular genotyping

Identification of MTB, and drug susceptibility testing to isoniazid (INH), streptomycin (SM), rifampicin (RMP), and ethambutol (EMB) were performed as reported before [22]. Beijing and non-Beijing strains were analyzed by single-nucleotide polymorphism (SNP) at position 779,615 [23] using real-time PCR with a TaqMan MGB probe [Primers, mtb779615-F: CGATTGGCCTGTGGTCACT; mtb779615-R: GAACAACAAGATCGCCTTCGA; Probe, wild-type (FAM): TAGGTGACCGTGTTGTC; mutant (VIC): TAGGTGACCGTCTTGTC]. Ancient and modern Beijing genotypes were identified by PCR, the conditions and analysis of which were described previously [24]. Spoligotyping was performed [25] in parallel and their genotypes were identified using the international MTB database (SpolDB4) [26].

Five different VNTR locus sets were tested for the identification of genotypic clusters: optimized-MIRU15-, optimized-MIRU24-, optimized-MIRU28-, JATA15-, and JATA18-VNTR sets (Supplementary Table S1). Amplified products were analyzed using a 3130 Genetic Analyzer (ABI) with the GeneMapper program, SV1210 microchip electrophoresis (Hitachi) or agarose gel electrophoresis. The copy number in each locus was calculated based on the molecular size of the PCR products and the number of tandem repeats in the genome of the H37Rv strain was used as the standard.

Genetically clustered strains were defined by the complete match of the VNTR profile. To confirm the appropriateness of each cluster, spoligotyping patterns were also considered. The proportion of clustered strains (the clustered proportion) was calculated using the "*n*" method, which is given by the number of isolates in clusters divided by the total number of isolates [18]. Polymorphic information content (PIC) was used as one of the estimators for the discriminatory power of typing loci [27]. Also, genetic diversity and discriminatory power were assessed by calculating the number of different VNTR patterns and the Hunter–Gaston discriminatory index (HGI) for each condition [28]. Using VNTR profiles of MTB isolates by optimized-MIRU15-, optimized-MIRU28-, JATA15-, and JATA18-VNTR systems, the minimum spanning trees were depicted using BioNumerics software version 4.61 (Applied Maths).

2.3. Statistical analysis

The chi-square test was used to compare the proportions between groups. Bonferroni's correction was used for multiple comparisons. Median and interquartile range (IQR) were presented for age distributions and the Kruskal–Wallis test was used to assess their possible differences among MTB subtypes. Polytomous logistic regression models for MTB lineages or sublineages as outcome variables were also used to investigate factors showing associations, after which adjusted odds ratios (aORs) and 95% confidence intervals (CIs) were calculated. Interaction terms were also considered when appropriate. Factors with biological meaning or showing $P < 0.2$ in univariate analysis were included in multivariate models. The McNemar's test was used to investigate a

possible inconsistency of the power to detect unique (non-clustered) strains between two VNTR sets, which tests whether the frequency of unique strains detected by one VNTR set is significantly different from that of another set. Statistical analysis was performed using Stata version 12 (Stata Corp, College Station, TX, USA), and $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Characteristics of the study population

For 465 MTB strains, the characteristics of the study population by MTB lineages, including Beijing sublineages, are provided in Supplementary Table S2. In summary, the median patient age was 39.0 years (IQR 29.2–50.6); 365 (78.5%) patients were male and 38 (8.2%) were HIV-positive.

3.2. Genotypes of MTB strains defined by SNPs and spoligotyping in Hanoi

Table 1 shows the proportions of ancient/modern Beijing and non-Beijing genotypes identified by the methods of SNPs and spoligotyping. Of the 465 strains tested, 175 (37.6%) belonged to the ancient Beijing sublineage and 97 (20.9%) were of the modern Beijing sublineage. Thus, the total proportion of the Beijing lineage strains was 58.5%. The third most prevalent type was East African Indian (EAI), which usually belongs to the Indo-oceanic lineage. Other spoligotypes, including H, LAM, T, U, and X, were seen in 65 cases (14.0%). In 37 cases (8.0%), spoligotypes were not registered in the SpolDB4 database. Thus, the spoligotyping method was limited in its ability to identify and classify genotypes.

When we tested another set of 223 DNA samples in Hanoi, the ancient Beijing sublineage was reproducibly predominant. Of these samples, 88 (39.4%) belonged to the ancient Beijing sublineage, 61 (27.4%) were of modern Beijing sublineage, and 74 (33.2%) were non-Beijing strains.

3.3. Patient characteristics stratified by ancient and modern Beijing sublineages, EAI genotype, and other strains

Ancient and modern Beijing genotype strains were both widespread among young patients [median age was 37.9 years (IQR, 29.2–49.1) and 34.8 years (IQR, 28.5–48.8), respectively], while EAI strains were frequently seen in a relatively older group [46.8 years (IQR, 33.1–56.3)] (Supplementary Table S2). The age distribution was significantly different among the four groups even after Bonferroni's correction (uncorrected $P = 0.0036$, Kruskal–Wallis test). When we investigated additional demographic parameters or clinical features such as lesions on chest radiography or HIV status by the chi-square test, the proportions of subcategories in each parameter or feature were not significantly different among the four groups (Supplementary Table S2).

Table 1
Frequency of the MTB genotype defined by SNPs and spoligotyping ($n = 465$).

Spoligotypes*	Frequency	Percentage
Beijing (ancient type)	175	37.6
Beijing (modern type)	97	20.9
EAI5	82	17.6
H1	3	0.7
H3	9	1.9
H4	1	0.2
LAM9	2	0.4
MANU2	1	0.2
S	2	0.4
T1	31	6.7
T2	4	0.9
T2-T3	4	0.9
T3	1	0.2
U	5	1.1
EAI4_VNM	9	1.9
X1	1	0.2
X2	1	0.2
Unknown	37	8.0

MTB: *Mycobacterium tuberculosis*.
* Beijing/non-Beijing and ancient/modern Beijing genotypes were classified by SNPs.

3.4. Genetic clustering based on VNTR typing methods using different locus sets

Because it is well known that the genetic clusters of Beijing genotypes are not clearly identified by the optimized-MIRU15- or MIRU24-VNTR typing systems [4], we added three different locus sets for the calculation of the clustered proportions: JATA15-and JATA18-VNTR locus sets and the recently proposed 24 plus 4 locus sets, optimized-MIRU28 system, considering clonal spread of Beijing genotypes.

Of the 465 strains, the proportions of clustered strains calculated from the optimized-MIRU15-, optimized-MIRU24-, and JATA15-VNTR sets were 259 (55.7%), 229 (49.2%), and 207 (44.5%), respectively. Because differences in these percentages are mainly attributed to the differences in discriminatory power to identify unique VNTR patterns, we compared the proportion of unique (nonclustered) strains detected by the JATA15 set and demonstrated that it was significantly higher than that of the MIRU15 or MIRU24 sets (data not shown; $P < 0.0001$ and $P = 0.0068$, respectively, by the McNemar's test). Based on the optimized-MIRU28 locus set, including three HV loci (VNTRs-3232, -3820, and -4120) [19], the proportion of clustered strains was 157 (33.8%), which was relatively lower than those of the aforementioned sets. As expected, the proportion calculated from the JATA18 set (JATA15 plus the same HV loci) was similarly low at 149 (32.0%) and not inferior to that of the MIRU28 system ($P = 0.1573$).

Ancient and modern Beijing genotype strains were more frequently clustered than the EAI genotype, even when considering multiple comparisons (uncorrected $P < 0.0001$, Supplementary Table S2). We also calculated the proportion clustered in each (sub)lineage using five different VNTR locus sets (Figure 1). In both the ancient and modern Beijing MTBs, the JATA sets tended to show a reduced proportion of clustered strains, presumably indicating high discriminatory power as compared with the MIRU systems using the equivalent number of VNTR loci. In contrast, this advantage of JATA sets was not observed for non-Beijing sublineages such as the EAI spoligotype (Figure 1). A similar tendency was obtained when the genetic diversity of MTB (sub)lineages and the discriminatory power of each VNTR set were assessed by the number of different VNTR patterns (=the number of unique strains plus one from each cluster), the proportion of unique strains, and the Hunter–Gaston Index (HGI) (Supplementary Table S3).

Because the discriminatory power of VNTR methods to assess MTB's clonality and transmission is largely affected by the MTB (sub)lineages analyzed, we used the minimum spanning tree program and further analyzed the relatedness of the Beijing and non-Beijing strains genotyped by the different locus sets (Supplementary Figure S1). Non-Beijing MTBs were subdivided into two large groups in both optimized-MIRU15- and optimized-MIRU28-VNTR systems. As compared with these MIRU systems, JATA15-and 18-VNTRs clearly separated the modern and ancient Beijing sublineages into two different groups, although these locus sets did not show a sufficient power to further divide non-Beijing MTBs.

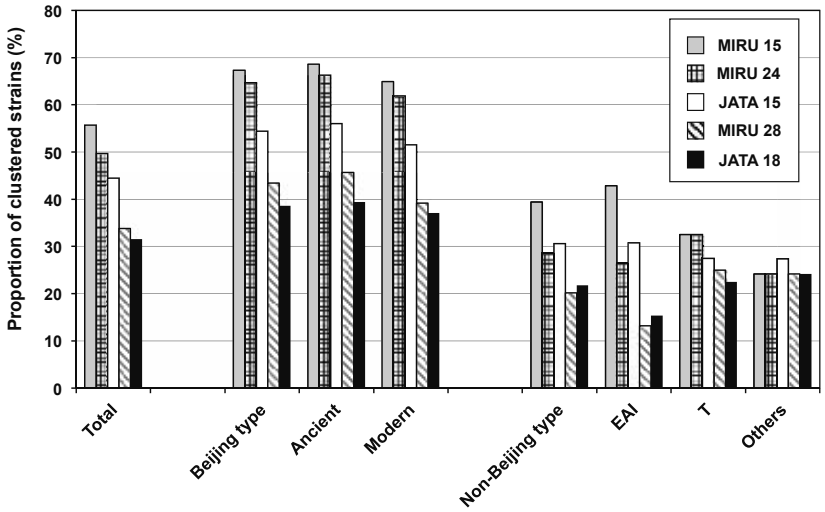

Figure 1. The proportion of clustered strains determined in each (sub)lineage using different VNTR locus sets. Using optimized-MIRU15, 24, 28, JATA15, and 18 locus sets, 465 MTB isolates were analyzed. The proportions of clustered strains were calculated.

3.5. Profiles of drug resistance harbored by ancient and modern Beijing sublineages, EAI genotype, and other strains

Next, we examined the relationship between drug resistance and the MTB genotypes. The proportions of strains carrying "any INH resistance" and "any SM resistance" tended to be high in the ancient Beijing genotype group, lower in the modern Beijing genotype group, and the lowest in the EAI genotype group. The differences in the proportion of INH- and SM-resistant strains among the four MTB genotype groups, including others, were statistically significant, even after considering multiple comparisons (uncorrected $P = 0.0001$ or <0.0001 by the chi-square test, respectively, Table 2). No significant difference was observed in the proportions of RMP, EMB, or multidrug resistance among these groups (Table 2).

Table 2
MTB sublineages and patterns of drug resistance ($n = 465$).

Drug resistance	Number (%) of isolates with drug resistance in the different groups of MTB genotype				
	Ancient Beijing $N = 175$	Modern Beijing $N = 97$	EAI $N = 91$	Others $N = 102$	P value*
Sensitive to all drugs	83 (47.4)	66 (68.0)	78 (85.7)	60 (58.8)	<0.0001
Any INH resistance	69 (39.4)	23 (23.7)	13 (14.3)	23 (22.6)	0.0001
Any RMP resistance	11 (6.3)	5 (5.2)	1 (1.1)	4 (3.9)	0.26
Any SM resistance	70 (40.0)	19 (20.0)	5 (5.5)	32 (31.4)	<0.0001
Any EMB resistance	5 (2.9)	2 (2.1)	0 (0.0)	4 (3.9)	0.307
INH monoresistance	21 (12.0)	11 (11.3)	8 (8.8)	8 (7.8)	0.671
RMP monoresistance	1 (0.6)	0 (0.0)	0 (0.0)	1 (1.0)	>0.999
SM monoresistance	21 (12.0)	8 (8.3)	0 (0.0)	18 (17.7)	0.0005
INH + SM	38 (21.7)	7 (7.2)	4 (4.4)	11 (10.8)	<0.0001
INH + EMB	0 (0.0)	0 (0.0)	0 (0.0)	1 (1.0)	0.624
SM + EMB	1 (0.6)	0 (0.0)	0 (0.0)	0 (0.0)	>0.999
INH + RMP	0 (0.0)	1 (1.0)	0 (0.0)	0 (0.0)	0.404
INH + RMP + SM	6 (3.4)	2 (2.1)	1 (1.1)	0 (0.0)	0.223
INH + RMP + EMB + SM	4 (2.3)	2 (2.1)	0 (0.0)	3 (2.9)	0.503

MTB: *Mycobacterium tuberculosis*; EAI: East African Indian; INH: isoniazid, RMP: rifampicin, SM: streptomycin, EMB: ethambutol.

* P value for an overall difference in the proportions of drug-resistant strains stratified by the four MTB subgroups was calculated using the chi-square test. Underlined values indicate that the statistical significance remained after Bonferroni's correction.

3.6. Characteristics associated with ancient and modern Beijing sublineages

To further clarify the phenotypic characteristics of ancient and modern Beijing sublineages, we compared them with non-Beijing strains, including EAI strains and others. By univariate analysis (Table 3), patients younger than 55 years old were associated with both ancient and modern Beijing strains. Relatively low BMI levels (16.0–18.4) also showed an association with ancient and modern Beijing genotypes. Herein, we used the optimized-MIRU28-VNTR system and defined the genetic clusters, which were strongly associated with ancient and modern Beijing strains. All the above

Table 3
Univariate analysis using polytomous logistic regression models to investigate factors possibly associated with MTB sublineages ($n = 465$).

	Odds ratio* (95% CI)	
	Ancient Beijing	Modern Beijing
Age: <55.0 years vs. ≥55.0 years	**3.38 (1.84-6.21)**	**2.66 (1.31-5.39)**
Female vs. male	1.17 (0.70–1.94)	1.46 (0.82–2.61)
Body mass index		
<16.0 vs. no undernutrition†	1.12 (0.60–2.10)	1.01 (0.46–2.24)
16.0–18.4 vs. no undernutrition	**1.60 (1.03-2.51)**	**1.85 (1.09-3.13)**
Living area		
New urban vs. suburban	0.99 (0.56–1.73)	0.61 (0.33–1.14)
Old urban vs. suburban	0.91 (0.50–1.63)	0.53 (0.27–1.02)
Smoking vs. nonsmoking	0.83 (0.54–1.29)	0.78 (0.47–1.31)
HIV (+) vs. HIV (−)	1.20 (0.56–2.56)	1.30 (0.54–3.12)
Cavity vs. no cavity on CXR	0.91 (0.58–1.43)	1.52 (0.85–2.71)
Infiltrates in ≥3 vs. <3 lung zones on CXR	1.29 (0.74–2.26)	1.31 (0.68–2.51)
Clustered vs. nonclustered§	**3.33 (2.10–5.27)**	**2.54 (1.48–4.36)**
INHr vs. INHs	**2.84 (1.77–4.55)**	1.36 (0.75–2.45)
SMr vs. SMs	**2.81 (1.76–4.49)**	1.03 (0.55–1.90)
RMPr vs. RMPs	2.52 (0.86–7.41)	2.04 (0.58–7.24)

MTB: *Mycobacterium tuberculosis*, HIV: Human immunodeficiency virus, CXR: chest radiography, INHr: resistant to isoniazid, INHs: sensitive to isoniazid, SMr: resistant to streptomycin, SMs: sensitive to streptomycin, RMPr: resistant to rifampicin, RMPs: sensitive to rifampicin, 95% CI: 95% confidence interval.
Boldfaced values indicate odds ratios and 95% CI with statistical significance ($P < 0.05$).

* The non-Beijing group including EAI strains was set as a reference.

† No undernutrition indicates a body mass index over 18.5.

§ Genetic clustering was defined on the basis of the optimized-MIRU28-VNTR system.

associations remained significant in multivariate analysis using the model including age, gender, BMI, presence of cavity on chest radiography, clustering status, and resistance to INH, SM, and RMP (Table 4).

We also analyzed the interaction term between genetic clustering and drug resistance and the independent effects of each category. By univariate analysis, INH resistance appeared to be associated with ancient Beijing strains (Table 3), but this association was lost after adjustment for age and other possible confounders (Table 4). The interaction term between INH resistance and genetic clustering did not show particular effects on ancient Beijing strains, whereas it showed significantly negative effects on modern Beijing strains, even after adjustment for possible confounders (Table 4).

SM resistance was also associated with the ancient Beijing strains in a univariate analysis (Table 3). This association was lost (aOR = 1.95, 95% CI 0.97–3.91) after adjustment for age and other possible confounders when genetic clustering was defined by optimized-MIRU28-VNTR (Table 4). When the clusters were defined by the JATA18-VNTR set, both INH and SM resistance showed weak but significant associations with the ancient Beijing strains after adjustment (aOR = 2.15, 95% CI 1.00–4.61 and aOR = 1.98, 95% CI 1.00–3.92, respectively) (data not shown). The interaction term between SM resistance and genetic clusters also tended to show slightly negative effects on modern Beijing strains, but it did not reach significant levels (Table 4). RMP resistance was not associated with either ancient or modern Beijing strains, and we could not attempt further analysis due to insufficient statistical power.

The relationship between BCG vaccination and the MTB subtype was not included in logistic regression analysis and was analyzed separately because of the large proportion of missing values. As a result, no significant associations were observed between these two factors (data not shown).

Table 4
Multivariate analysis using polytomous logistic regression models to investigate factors possibly associated with MTB sublineages ($n = 465$).

	Odds ratio* (95% CI)	
	Ancient Beijing	Modern Beijing
Age: <55.0 years vs. ≥55.0 years	**3.44 (1.70–6.98)**	**2.19 (1.03–4.66)**
Female vs. male	1.42 (0.79–2.55)	1.62 (084–3.10)
Body mass index:		
<16.0 vs. no undernutrition†	1.32 (0.64–2.73)	0.91 (0.38–2.19)
16.0–18.4 vs. no undernutrition	**1.86 (1.13–3.07)**	**2.00 (1.13–3.54)**
Cavity vs. no cavity on CXR	0.87 (0.53–1.42)	1.44 (0.78–2.64)
Clustered vs. nonclustered§	**3.28 (1.70–6.33)**	**4.32 (2.17–8.62)**
INHr vs. INHs	1.83 (0.85–3.92)	2.03 (0.84–4.89)
SMr vs. SMs	1.95 (0.97–3.91)	0.93 (0.39–2.24)
RMPr vs. RMPs	1.11 (0.32–3.85)	2.11 (0.49–9.09)
Interaction between clustering and INHr**	0.44 (0.13–1.46)	**0.15 (0.03–0.66)**
Interaction between clustering and SMr**	1.23 (0.37–4.13)	0.56 (0.11–2.91)

MTB: *Mycobacterium tuberculosis*, CXR: chest radiography, INHr: resistant to isoniazid, INHs: sensitive to isoniazid, SMr: resistant to streptomycin, SMs: sensitive to streptomycin, RMPr: resistant to rifampicin, RMPs: sensitive to rifampicin, 95% CI: 95% confidence interval. For assessment of the interaction between clustering and drug resistance, the main effects with an interaction term were included in a polytomous logistic regression model.
Boldfaced values indicate odds ratios and 95% CI with statistical significance ($P < 0.05$).
* The non-Beijing group including EAI strains was set as the reference.
† No undernutrition indicates a body mass index over 18.5.
§ Genetic clustering was defined on the basis of the optimized-MIRU28-VNTR system.
** A full factorial model was developed; both interaction terms and independent effects are shown.

4. Discussion

Our study showed that MTB strains of ancient and modern Beijing genotypes consisted of the largest and the second largest groups circulating among patients newly diagnosed with smear-positive, culture-positive pulmonary TB in Hanoi, Vietnam. Age distribution, genetic clustering, and the patterns of primary drug resistance were differently dependent on MTB genotypes, including Beijing sublineages. This was the first study in the northern part of Vietnam that investigated the phenotypic characteristics of Beijing sublineages.

In our study population, Beijing genotype strains accounted for 58.5% of MTB strains, comparable with that of East Asian areas [26]. This prevalence is higher than that reported from rural Vietnam, where EAI strains are more predominant [29]. EAI belongs to the Indo-oceanic lineage, one of the most ancestral of the seven MTB lineages [2]. EAI strains may have originated from Africa [30] and spread to the Southeast Asian area accompanied by the population movement through the southern regions of Eurasia. These strains may have gradually been replaced by the recent expansion of the Beijing genotype strains [29]. This hypothesis is worth considering and should be tested by monitoring MTB (sub)lineage distribution in Hanoi for an extended timeframe. A long-term study is required because the Beijing genotype is more commonly seen in younger populations and is clustered compared with those infected with the EAI genotype and others in this study area; this suggests the possibility of recent spread of the Beijing genotype.

Of the Beijing genotype strains in Hanoi, located in the northern part of Vietnam, the ancient sublineage accounted for two-thirds and the modern sublineage one-third. Interestingly, this distribution pattern is similar to that from Japan [10,11] and Korea [12], but this pattern is different from the patterns reported from most other parts of the world, such as China [31], Russia [32], South Africa [9], and Europe [16], where the modern Beijing genotype represents 65%–95% of Beijing strains. Another study from Ho Chi Minh city in southern Vietnam also has showed that the modern Beijing genotype is observed three times more frequently than the ancient Beijing subtype [16]. This difference in distribution between these two major cities at the far ends of Vietnam may be due to the northern part of Vietnam bordering on southern China, where ancient Beijing is also more frequently found as compared with the northern areas of China [31]. In addition, in our study population, the proportions of both the ancient and modern Beijing sublineages were higher in younger patients, suggesting their recent dissemination. This finding is in contrast to the ancient type spreading among older patients in the southern part of Vietnam [16]. Further information regarding sublineage distribution throughout many Asian countries is necessary to approach the evolutionary history, including a potential branching point between the ancient and modern Beijing genotypes. Thus far, little information regarding these sublineages is available in Southeast Asia, including Vietnam [5].

Because Beijing genotype isolates are closely genetically related to each other, many genotyping methods exhibit low discriminatory power and a limited potential to assess their genetic clonality that reflects epidemiological transmission [19,33]. In our tested population, the discriminatory power of JATA15 (a local Japanese system used in a Beijing genotype-predominant area) was higher than that of optimized-MIRU15 or 24, in which all worldwide lineages are the targets. When appropriate HV loci were added to either the optimized-MIRU24 or JATA15 set, the genotyping systems were more suitable for the Beijing family. Considering the resource-poor settings in many Asian countries, however, it is difficult to analyze more than 20 genetic loci for domestic public health problems with the exception of international research

activities. For instance, in Japan, 70%–80% of MTB isolates are of the Beijing genotype [11], and 12 or 15 VNTR loci have been preferred on site [20]. A similar cost-effective VNTR locus-set has also been recommended in China [34]. Despite the relatively small number of tested loci, in our study, the Japanese system had high discriminatory power for MTBs in the northern part of Vietnam. PIC for one of the HV loci, VNTR-4120, in Japan and Thailand was reportedly 0.90 and 0.58, respectively [27,35]. In our study, PIC for the VNTR-4120 locus in Hanoi was 0.83 (data not shown). This finding may indicate that the distribution patterns of the Beijing MTB genotypes in Hanoi resemble those in Tokyo, including both the ancient and modern sublineages. Considering the proportion of unique (non-clustered) strains and other indexes, including HGI, it appears that MIRU28 and JATA18 have a higher discriminatory power than the others. However, a drawback of adding the HV loci is that a large number of nucleotide repeats in the loci should be distinguished using a high-resolution genetic analyzer. Although it is conceivable that TB transmission was ongoing during the study period in Hanoi districts in which patients were recruited, direct information regarding transmission chains between clustered cases or the possible involvement of outbreak strains was not available, which is a limitation of our study. Minimum essential VNTR loci optimal to TB transmission should be further examined in a prospective population-based study and discussed with information about the epidemiological link.

Even when we used VNTR locus sets with a high discriminatory power, Beijing genotype strains were frequently clustered, whereas the majority of the EAI genotype and other strains were observed as nonclustered strains. The associations between the Beijing sublineages and clustering remained significant, even after adjustment for other factors in a polytomous regression model. In our study area, the modern Beijing genotype strains were less prevalent than the ancient Beijing genotype strains, while the proportion of clustered strains belonging to the modern Beijing genotype was comparable to that of the ancient Beijing genotype, irrespective of the different VNTR loci sets. Although we have no direct evidence, the modern Beijing strains may have the potential to spread further in this area. Indeed, previous reports have often shown that these strains have a high transmissibility [36,37].

Associations between the antibiotic resistance and Beijing genotype strains have also been investigated in many studies in various geographical settings [3–5], although the results are controversial. Interestingly, the interaction between INH resistance and genetic clustering was significantly less likely to occur in the modern Beijing strains, irrespective of possible confounders in our study. Although Beijing genotype strains are often spreading as MDR-TB (INH and RMP resistance) in many areas worldwide, the clustered modern Beijing genotype strains identified in the Hanoi area may belong to some different subgroup(s) with a tendency to spread without harboring INH-resistance. One possibility is that these strains may be disadvantageous to propagation once they acquire drug resistance, bearing a higher "fitness cost" than those widely spreading in other areas. A detailed comparative analysis is necessary to better understand this issue, possibly analyzing genome-wide variations among several subgroups of the modern Beijing sublineage. Another possibility is that a majority of modern Beijing sublineage strains in Hanoi may have recently entered across neighboring countries as drug-susceptible strains and may currently be spreading in Hanoi.

Also, in our study, the ancient Beijing strains in Hanoi tended to carry INH and SM resistance more frequently than the modern Beijing strains and others. However, this association was not always significant, but it was affected by other factors such as the age of the patients in the multivariate analysis. The tendency of the ancient Beijing strains to carry drug resistance has also been demonstrated in a few reports from East Asian areas where the Beijing strains are predominant [21,38]. However, the drug resistance patterns of the ancient Beijing genotype strains were different: INH and RMP in one report [21] and pyrazinamide and RMP in another [38]. These differences may be relevant to the history of when the antibiotics were introduced or because of other confounders. In Vietnam, SM was initially used for the treatment of wound infections during the war in the early 1950s, after which INH was widely implemented for tuberculosis treatment, which may partly explain the current spread of SM- and INH-resistant strains. Depending on drug resistance, the fitness of the ancient Beijing genotype strains may be retained or may even be stronger than the modern Beijing strains. We revealed that 116 (84.1%) of 138 INH-resistant strains identified in this study harbored a single *katG* S315T mutation (unpublished data), which seems to have a negligible fitness cost, thus indicating no reduction in transmissibility [39,40]. Further study is necessary to elucidate whether bacterial genetics have an epistatic impact on propagation of drug resistance through the genotype to which they belong [39].

Both ancient and modern Beijing strains were more likely to be detected from relatively undernourished patients (~60%), whereas more than half of EAI strains were observed in patients with normal BMI. This association remained significant even after adjustment for possible confounders. Severe undernutrition with a BMI less than 16.0 did not show significant association, probably due to the small number of cases or different reasons. Malnutrition itself may be a condition that makes patients vulnerable to infections by the Beijing genotype strains, or it may be brought on by infection with the MTB strains [41]. The relationship between host nutritional state and activation of modern/ancestral MTB lineages would be one of the important topics to consider in the host–pathogen interaction and future therapeutic modalities.

Although it is difficult to adjust for the historical flow of MTB strains introduced from outside areas, potential confounders to the interpretation of the genotype–phenotype relationship of the MTB strains were minimized in our study. Both the ancient and modern Beijing genotype strains were commonly observed with non-Beijing strains among the Vietnamese population with relatively homogeneous ethnicity [42]. This indicates that the northern part of Vietnam may be one of the suitable geographic areas to characterize these Beijing sublineages as compared with the non-Beijing strains.

In conclusion, our study showed that among patients newly diagnosed with smear-positive, culture-positive pulmonary TB in Hanoi, Vietnam, ancient Beijing genotype strains are predominant, followed by the modern Beijing sublineage. Both appear to be currently spreading; however, their phenotypes are different, even though they both belong to the same Beijing family. Our findings may provide an insight into the reason(s) for inconsistencies among previous results regarding the overall phenotypic characteristics of the Beijing family.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.tube.2014.09.005.

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Age-dependent association of mannose-binding lectin polymorphisms with the development of pulmonary tuberculosis in Viet Nam

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ABSTRACT

Mannose-binding lectin (MBL) binds to pathogens and induces complement-mediated opsonophagocytosis. Although the association between *MBL2* polymorphisms and tuberculosis (TB) has been studied in various populations, the results are controversial. We explored the stages of TB associated with *MBL2* polymorphisms. *X*/*Y* (rs7096206) and *A*/*B* (rs1800450) were genotyped in 765 new patients with active pulmonary TB without HIV infection and 556 controls in Hanoi, Viet Nam. The *MBL2* nucleotide sequences were further analyzed, and plasma MBL levels were measured in 109 apparently healthy healthcare workers and 65 patients with TB. Latent TB infection (LTBI) was detected by interferon-gamma release assay (IGRA). The *YA*/*YA* diplotype, which exhibited high plasma MBL levels, was associated with protection against active TB in younger patients (mean age = 32) ≦ 45 years old (odds ratio, 0.61; 95% confidence interval, 0.46–0.80). The resistant diplotype was less frequently found in the younger patients at diagnosis ($P = 0.0021$). *MBL2* diplotype frequencies and plasma MBL levels were not significantly different between the IGRA-positive and -negative groups. *MBL2 YA*/*YA* exhibited a protective role against the development of TB in younger patients, whereas the *MBL2* genotype and MBL levels were not associated with LTBI. High MBL levels may protect against the early development of pulmonary TB after infection.

1. Introduction

Mycobacterium tuberculosis (*Mtb*) is the causative agent of tuberculosis (TB) in humans and presumably infects a third of the world's population. *Mtb* establishes a persistent infection in immune cells such as macrophages, and 5–10% of immunocompetent individuals develop active TB during their lifetime, whereas the others limit infection by successful containment of *Mtb* in granulomas. The innate immune response induces activation of the T helper 1 (Th1)-type immune system and plays an important role in host defense against the development of TB [1]. Many studies have reported the association between TB and polymorphisms of host genes related to innate immunity [2].

Mannose-binding lectin (MBL) is an acute-phase serum protein in the collectin family that recognizes a pathogen by its carbohydrate-recognition domains [3]. MBL is synthesized in the liver and circulates in the form of oligomers complexed with MBL-associated serine proteases (MASPs). Upon binding to the sugar moieties on the pathogen surface, MASPs are activated to initiate the lectin pathway of complement activation, which results in opsonization and phagocytosis or lysis of microorganisms. Besides its direct action as an opsonin and its key role in the lectin pathway, MBL may modulate inflammatory responses and immune activation [4].

Abbreviation: Mtb, Mycobacterium tuberculosis.

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MBL is encoded by *MBL2*, which is located on chromosome 10, and six *MBL2* single nucleotide polymorphisms (SNPs) are associated with serum levels and/or functions of MBL. Three nonsynonymous nucleotide substitutions in exon 1 change the wild *A* allele to the three variant alleles (*A*/*B*, *A*/*C*, and *A*/*D*), which disrupt the collagenous structure and the formation of functional oligomers. The other alleles, *H*/*L*, *X*/*Y*, and *P*/*Q*, are distinguished by the SNPs in the promoter and 5′-untranslated regions, and the *X* allele shows the lowest transcriptional activity among them [5]. Because of strong linkage disequilibrium, seven haplotypes are commonly observed and often classified into three groups of higher producing (*HYPA*, *LYPA*, and *LYQA*), lower producing (*LXPA*), and nonfunctional (*LYPB*, *LYQC*, and *HYPD*) haplotypes.

These genetic variations that result in MBL deficiency are associated with a wide variety of diseases, including respiratory tract infections, presumably because of the leak of circulating MBL into inflamed airways [6]. However, *MBL2* polymorphisms show conflicting results and confer either resistance or susceptibility toward pulmonary TB [2]. According to some studies, MBL deficiency is associated with protection against TB disease, raising the hypothesis that the uptake of microorganisms by phagocytes is enhanced by MBL binding, which results in the promotion of infection by intracellular pathogens [7,8]. In contrast, other investigators have suggested that high MBL levels have a protective effect against TB [9,10].

Immune responses control *Mtb* infection in the latent phase, but *Mtb* is reactivated from an immunological equilibrium to develop TB disease [11]. The persistence of the latency period in adult patients with TB varies greatly among individuals, and this may reflect the duration of successful *Mtb* containment. During this process, it is believed that pathogenic factors, including different genotype strains, may also play a role [12]. We found a protective role of the interferon gamma receptor 2 gene (*IFNGR2*) polymorphism against TB; furthermore, we found that the resistant alleles tended to be less frequent in younger patients at diagnosis when we investigated polymorphisms in the Th1-immune response genes in Vietnamese patients with TB [13]. Grant et al. also found an age-dependent association of thymocyte selection-associated high mobility group box gene (*TOX*) variants in Morocco and Madagascar and highlighted the importance of age at TB diagnosis, which is correlated with the duration of the latency period in endemic areas [14]. The inconsistent association between *MBL2* and TB in different studies may be attributable to the different stages of *Mtb* infection from latent TB infection (LTBI) to TB disease; therefore, in the present study, we explored whether *MBL2* polymorphisms or MBL levels are associated with the development of active TB in apparently immunocompetent patients of various ages or the stage of LTBI in Viet Nam, a country with high TB prevalence.

2. Materials and methods

2.1. Study population

The patients and controls were recruited from Hanoi, Viet Nam [13,15,16]. In total, 832 patients (age, 41 ± 14.4 years; 77.6% males) without a previous TB episode were recruited immediately after the diagnosis of new smear-positive pulmonary TB was made. Pulmonary physicians treated them with anti-TB drugs according to the guidelines of the national TB program. Fifty-three HIV-positive patients with TB, four with no information about HIV status, and nine with missing age data were excluded from further analysis. *Mtb* genotyping method was described elsewhere [16]. Beijing genotype of *Mtb* isolates was distinguished from non-Beijing genotype in 429 TB patients with no HIV infection.

The control group for this genetic association study consisted of 556 healthy volunteers (age, 36 ± 10.3 years; 48.6% males) who had the same ethnicity and were residents in the same area of Hanoi city. Information of their LTBI status was not available, but 109 disease-free healthcare workers (HCWs; age, 34 ± 10.1 years; 23.9% males) were also recruited and their LTBI status was assessed by an enzyme-linked immunosorbent assay (ELISA)-based interferon gamma release assay (IGRA; QuantiFERON-TB Gold In-Tube™, Cellestis, Victoria, Australia) [17]. All were unrelated Vietnamese. Informed consent was obtained from all participants. The study protocol was approved by the ethics committees of the Ministry of Health, Viet Nam (4481/QD-BYT, 2529/QD-SYT), the National Center for Global Health and Medicine (NCGM-A-000185-00, 63), and the Research Institute of Tuberculosis (RIT/IRB25-1, 25-2), Japan.

2.2. Haplotype analysis of MBL2 SNPs in the Vietnamese HCWs and patients with TB

Genomic DNA samples from the 109 HCWs and 156 patients with TB were randomly subjected to polymerase chain reaction (PCR) amplification of the *MBL2* promoter and exon 1 regions with the primers 5′-GACCTATGGGGCTAGGCTGCTGAG-3′ and 5′-CCCCAGGCAGTTTCCTCTGGAAGG-3′ using TaKaRa LA Taq (TaKaRa, Shiga, Japan). The amplified products (1112 bp) were purified and sequenced with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) using the 3130xl Genetic Analyzer (Applied Biosystems). The synonymous SNP in exon 4 (rs930507) was amplified by PCR with the primers 5′-CTTTGTACCAGTCTGTCTGTTCAC-3′ and 5′-GGCCTGGAACTTGACACACAAGGC-3′ and genotyped using the restriction fragment length polymorphism method with *Ban* II (TaKaRa).

2.3. Plasma MBL level assay

Plasma MBL levels in samples were assayed by ELISA (Human MBL Quantikine ELISA Kit; R & D Systems, Minneapolis, MN, USA), which can specifically detect oligomeric forms of natural human MBL in serum, heparinized plasma, and EDTA plasma samples. Whole blood was divided into an EDTA tube and a negative control tube for the IGRA (nil tube). Plasma was separated immediately from the EDTA tube, whereas the nil tube was incubated at 37 °C for 16–24 h and centrifuged to separate the plasma. MBL levels in the plasma from the two procedures were compared for 31 individuals, and the coefficient of variation was calculated as 13.2%. Because incubation with heparin did not affect the results considerably, MBL levels were assayed in plasma supernatants from the control tubes for 109 HCWs and 65 patients with TB before the initiation of anti-TB treatment (0 month), after the initial phase of treatment (2 months), and at the end of treatment (7 months). These subjects were selected randomly from the abovementioned 156 patients.

2.4. MBL2 X/Y and A/B genotyping

X/*Y* (rs7096206) and *A*/*B* (rs1800450) polymorphisms were amplified in one DNA fragment by PCR using primers 5′-ACCTGGGTTTCCACTCATTCTCAT-3′ and 5′-CCCCAGGCAGTTTCCTCTGGAAGG-3′. An amplified product of 623 bp was digested with *Btg* I (New England Biolabs, Ipswich, MA, USA) to genotype *X*/*Y* or with *Ban* I (New England Biolabs) to genotype *A*/*B*, and they were electrophoresed on 2% agarose gels with ethidium bromide. Genotypes were determined by the length of the digested PCR products (*Y* allele with 540 bp after *Btg* I digestion and *A* allele with 536 bp after *Ban* I digestion).

2.5. Statistical analysis

Frequencies of haplotypes containing multiple polymorphic sites were estimated by Haploview ver. 4.2 [18]. Each individual's haplotypes were estimated by the PHASE program v.2.1 [19]. The association between the *MBL2* polymorphisms and plasma MBL levels after logarithmic transformation was analyzed using a multiple regression model. Differences in levels among the *MBL2* diplotype-based groups were further assessed by one-way analysis of variance (ANOVA) with the Tukey–Kramer method. Hardy–Weinberg exact tests were conducted to examine whether the genotype frequencies in the populations were compatible with Hardy–Weinberg equilibrium. TB development associated with a particular *MBL2* diplotype was assessed by odds ratios (ORs) and 95% confidence intervals (CIs) using a logistic regression model in which the interaction term between a particular MBL diplotype and age was also considered. The age-dependent trend for the presence of the *MBL2* diplotype in the patient population and their subgroups divided by *Mtb* genotypes was assessed by the change in odds at the 10-year interval in another logistic model.

Plasma MBL levels between groups with and without LTBI, as estimated by the IGRA results, were compared by analysis of covariance after adjusting for age and gender. The overall effects of *MBL2* diplotype, age, and gender on plasma MBL levels throughout the anti-TB treatment period were assessed using a random intercept model. All statistical analyses were performed using JMP ver. 9.0.0 (SAS Institute, Cary, NC, USA). Stata ver. 11 (StataCorp, College Station, TX, USA) was also used for the random intercept model analysis. *P*-values of <0.05 were considered statistically significant.

3. Results

3.1. Distribution of MBL2 haplotypes in the Vietnamese population

The *HYPA*, *LYPA*, *LYQA*, *LXPA*, and *LYPB* haplotypes in the Vietnamese HCWs and TB patients were estimated by the PHASE program (Table 1). The *LYQC* and *HYPD* haplotypes were not found. Thus the five haplotypes were observed in the Vietnamese population and they were identical to those directly determined by long-range PCR from other Asian populations [20,21]. Promoter −503 A/G SNP (rs7100749) was also polymorphic in the Vietnamese population; the −503 A allele was strongly associated with the *LYPA* haplotype. No novel mutation was found in the 156 patients with TB, including the randomly selected 65 individuals whose plasma MBL levels were measured afterwards in this study. Exon 4 synonymous C/G SNP (rs930507) is known to affect the MBL level of *LXPA* [22], but the *LXPA-rs930507 G* haplotype was rarely found; the estimated frequency was 0.02 in the Vietnamese population. Therefore, rs930507 was excluded from further analysis.

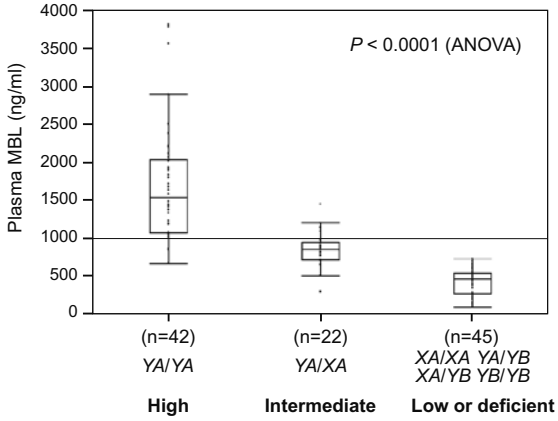

Fig. 1. Mannose-binding lectin *(MBL)2* diplotypes and plasma MBL levels in healthcare workers. Box-and-whisker plots of plasma MBL levels according to *MBL2* diplotypes of *X*/*Y* and *A*/*B* polymorphisms in healthcare workers. Each dot represents an individual. Differences in levels among *MBL2* diplotype-based groups were assessed by one-way analysis of variance.

3.2. MBL2 haplotypes associated with plasma MBL levels in the population

In agreement with previous studies [3], *X*/*Y* and *A*/*B* polymorphisms were strong determinants of plasma MBL levels in the multiple regression model ($P < 0.0001$ and $P < 0.0001$). *H*/*L* was only weakly associated with MBL in the same model ($P = 0.0378$). Neither *P*/*Q* nor age was associated with MBL levels in these 109 Vietnamese HCWs ($P = 0.1566$ and $P = 0.2484$). On the basis of these findings, the *MBL2* diplotypes were divided into three groups according to MBL levels, i.e., high (*YA*/*YA*), intermediate (*XA*/*YA*), and low or deficient (*XA*/*XA*, *YA*/*YB*, *XA*/*YB*, and *YB*/*YB*; Fig. 1). MBL levels were actually different among the three groups ($P < 0.0001$ by ANOVA), and the difference was significant between any two of the three groups (data not shown). The plasma level that defines MBL deficiency has not been clearly determined, but a recent study with a large sample size ($n = 1037$) using the same ELISA kit adopted ⩽1000 ng/ml as the category for partial and severe MBL deficiency [23]. Our classification almost matched with this definition, as MBL levels were >1000 ng/ml in 38/42 (90.5%) of the *YA*/*YA*-carrying individuals and ⩽1000 ng/ml in 45/45 (100.0%) of the *XA*/*XA*- or *B*-carrying individuals.

3.3. YA/YA diplotype associated with protection against TB in younger patients

Because plasma MBL levels largely depended on the combinations *X*/*Y* and *A*/*B*, frequencies of these variants were compared between all patients with TB and the controls. The genotype

Table 1
Estimated haplotypes and their frequencies in the Vietnamese healthcare workers ($n = 109$) and TB patients ($n = 156$).

	Promoter								Exon1		HCWs		TB patients	
	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	*n*	Frequency	*n*	Frequency
Haplotype	−550 *H*/*L*						−221 *X*/*Y*		+4 *P*/*Q*	G54D *A*/*B*				
HYPA	G	G	A	A	A	ins	G	C	C	G	97	0.445	136	0.436
LYPA	C	A	A	A	A	ins	G	C	C	G	16	0.064	24	0.077
LYQA	C	G	C	G	G	del	G	T	T	G	21	0.096	26	0.083
LXPA	C	G	A	A	A	ins	C	C	C	G	46	0.211	74	0.237
LYPB	C	G	A	A	A	ins	G	C	C	A	38	0.174	52	0.167

(1) rs11003125, (2) rs7100749, (3) rs11003124, (4) rs7084554, (5) rs36014597, (6) rs10556764, (7) rs7096206, (8) rs11003123, (9) rs7095891, (10) rs1800451.
Abbreviations: ins, insertion; del, deletion.
Statistically significant difference between the haplotype frequencies of 156 TB patients and those of 109 HCWs was not observed.

Table 2
MBL2 diplotypes in controls and patients with TB.

MBL levels	Diplotype	Full population					⩽45 years					>45 years				
		Controls (n = 556)		TB (n = 765)		OR (95% CI)	Controls (n = 436)		TB (n = 457)		OR (95% CI)	Controls (n = 120)		TB (n = 308)		OR (95% CI)
		No.	(%)	No.	(%)	[P value]	No.	(%)	No.	(%)	[P value]	No.	(%)	No.	(%)	[P value]
High	*YA*/*YA*	224	(40.3)	262	(34.2)	**0.77 (0.62–0.97) [P = 0.028]**	178	(40.8)	135	(29.5)	**0.61 (0.46–0.80) [P = 0.0004]**	46	(38.3)	127	(41.2)	1.13 (0.73–1.74) [P = 0.66]
Intermediate	*YA*/*XA*	148	(26.6)	231	(30.2)		122	(28.0)	154	(33.7)		26	(21.7)	77	(25.0)	
Low	*XA*/*XA*	35	(6.3)	50	(6.5)		23	(5.3)	30	(6.6)		12	(10.0)	20	(6.5)	
	YA/*YB*	98	(17.6)	142	(18.6)		76	(17.4)	91	(19.9)		22	(18.3)	51	(16.6)	
	XA/*YB*	33	(5.9)	56	(7.3)		24	(5.5)	34	(7.4)		9	(7.5)	22	(7.1)	
	YB/*YB*	18	(3.2)	24	(3.1)		13	(3.0)	13	(2.8)		5	(4.2)	11	(3.6)	

TB development associated with *YA*/*YA* was assessed by odds ratios (OR) with non-*YA*/*YA* as a reference. *P* values and ORs with confidence intervals (CIs) shown in bold type are statistically significant.

Table 3
Tendency for the presence of high or low MBL level diplotypes in the order of age strata: subgroup analysis by *Mtb* strains.

Age (years)	TB (n = 765)	(%)	Beijing (n = 250)	(%)	Non-Beijing (n = 179)	(%)
High MBL level (*YA*/*YA*) diplotype (n/N)						
Total	262/765	(34.2)	87/250	(34.8)	69/179	(38.5)
16–25	34/122	(27.9)	10/39	(25.6)	9/28	(32.1)
26–35	51/168	(30.4)	19/68	(27.9)	7/32	(21.9)
36–45	50/167	(29.9)	19/53	(35.8)	9/29	(31.0)
46–55	73/180	(40.6)	27/64	(42.2)	26/47	(55.3)
56–65	33/81	(40.7)	7/19	(36.8)	10/24	(41.7)
66-	21/47	(44.7)	5/7	(71.4)	8/19	(42.1)
OR per 10-year change (95% CI)	**0.85 (0.76–0.94)**		**0.77 (0.63–0.94)**		**0.82 (0.67–0.99)**	
Low MBL levels (*XA*/*XA* and *YB*-positive) diplotypes (n/N)						
Total	272/765	(35.6)	85/250	(34.0)	67/179	(37.4)
16–25	50/122	(41.0)	19/39	(48.7)	12/28	(42.9)
26–35	58/168	(34.5)	27/68	(39.7)	9/32	(28.1)
36–45	60/167	(35.9)	16/53	(30.2)	13/29	(44.8)
46–55	59/180	(32.8)	21/64	(32.8)	14/47	(29.8)
56–65	27/81	(33.3)	2/19	(10.5)	12/24	(50.0)
66-	18/47	(38.3)	0/7	(0.0)	7/19	(36.8)
OR per 10-year change (95% CI)	1.05 (0.94–1.16)		**1.43 (1.16–1.78)**		0.98 (0.81–1.19)	

The trend for the presence of the corresponding diplotype was calculated as an odds ratio in a logistic model when the patients were 10 years younger at the time of diagnosis. The corresponding *P*-values are also shown in the Section 3. The bold font denotes statistically significant odds ratio (OR); CI, confidence interval. The frequencies of diplotypes in TB patients with Beijing genotype and with non-Beijing genotype are shown in Supplementary Table.

distribution of the *X*/*Y* and *A*/*B* polymorphisms did not deviate from the Hardy–Weinberg equilibrium. We found that *YA*/*YA* was significantly associated with protection against TB (P = 0.028, OR, 0.77; 95% CI, 0.62–0.97; Table 2), even after adjustment for gender (data not shown). When *YA*/*YA* was further analyzed together with age (⩽45 years or >45 years), the genotype–age interaction was significant (P for interaction = 0.018). Therefore, the patients with TB and the corresponding controls were divided into two subgroups. A significant negative association was observed between TB and *YA*/*YA* only in the subgroup (mean age = 32) equal to or younger than 45 years old (P = 0.0004, OR, 0.61; 95% CI, 0.46–0.80; Table 2).

Considering the importance of the genotype–age interaction in TB pathogenesis, we investigated the age-dependence of the *YA*/*YA* frequencies in the patient group. When the patients were 10 years younger at the time of diagnosis, the trend of carrying *YA*/*YA* was significantly lower (P = 0.0021; Table 3), whereas such age-dependency was not observed in the control subjects (data not shown). The frequencies of *B*-allele-positive or *XA*/*XA* diplotypes resulting in low or deficient MBL levels were not significantly different between patients and controls, regardless of age stratification (Table 3).

Genetic information about the pathogen was available for 429 patients with TB among the HIV-negative cases. Beijing genotype *Mtb* strains are known to infect Asians and are spreading worldwide [24]. These strains were isolated from 250 patients in our Vietnamese study, and non-Beijing strains were found in 179 patients [16]. Regardless of *Mtb* genotype, the *YA*/*YA*-resistant diplotype was significantly less frequent when patients were 10 years younger at the time of diagnosis (P = 0.02 in the Beijing strain group and P = 0.04 in the non-Beijing strain group; Table 3), whereas low-level or deficient *MBL2* diplotypes containing the *B*-allele or *XA*/*XA* were more frequent in the younger age group only among patients with TB of the Beijing genotype strains (P = 0.0014; Table 3). When clinical information from the 506 patients with TB was analyzed, *MBL2* variations were not associated with TB severity, as estimated by the area of lung infiltration or recurrence of TB after therapy (data not shown).

3.4. Plasma MBL levels and diplotype frequencies not associated with the status of LTBI

Among the 109 HCWs, 68 (age, 33 ± 9.5 years; 22.1% males) were IGRA-negative while 41 (age, 35 ± 11.1 years, 26.8% males) were IGRA-positive. Plasma MBL levels and *MBL2* diplotype frequencies were not significantly different between IGRA-positive and -negative HCWs (Fig. 2), even after adjusting for age and gender (data not shown).

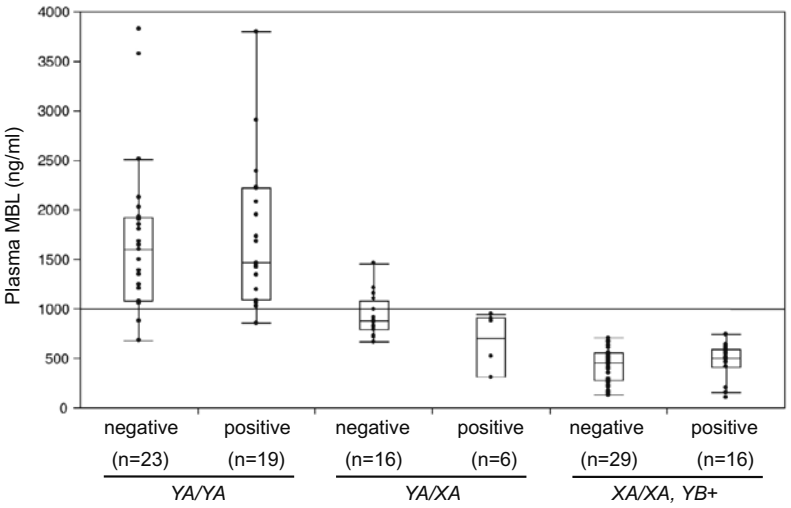

Fig. 2. Plasma mannose-binding lectin (MBL) levels in healthcare workers with and without latent tuberculosis infection (LTBI). Box-and-whisker plots of plasma MBL levels according to interferon gamma release assay (IGRA)-positive and -negative healthcare workers. Each dot represents an individual. Plasma MBL levels between groups were compared after adjusting for age and gender by analysis of covariance.

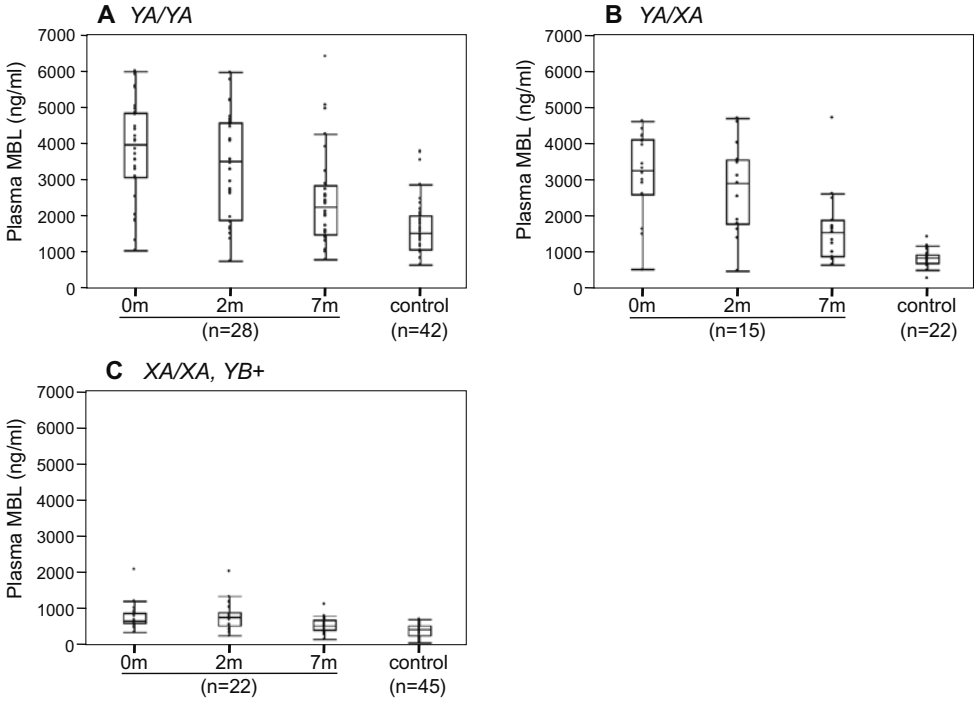

Fig. 3. Time-dependent changes in plasma mannose-binding lectin (MBL) levels in patients with tuberculosis (TB). Box-and-whisker plots of plasma MBL levels from the patients with TB [before (0 month), during (2 months), and at the end of (7 months) treatment] and from healthcare workers (control) with the *YA*/*YA* diplotype and high MBL levels (A), *YA*/*XA* diplotype with intermediate MBL levels (B), and *XA*/*XA* and YB-positive diplotypes with low or absent MBL levels (C). Each dot represents an individual. The overall effects of *MBL2* diplotype, age, and gender on plasma MBL levels throughout the anti-TB treatment period were assessed by using a random intercept model.

3.5. MBL diplotype-dependent plasma MBL levels and their changes during anti-TB treatment

MBL is an acute-phase reactant protein [25,26], and its plasma level is elevated in patients with TB [9]. We assayed MBL levels in serial plasma samples from 65 patients with TB [before (0 month), during (2 months), and at the end of (7 months) anti-TB treatment]. Plasma MBL levels were the highest before treatment and decreased during treatment (Fig. 3). MBL levels were significantly affected by *MBL2* diplotype throughout the treatment course ($P < 0.0001$), whereas age and gender did not affect the MBL levels ($P = 0.452$ and $P = 0.866$). The MBL concentrations at 7 months were still significantly higher than those of controls in any diplotype groups.

4. Discussion

We found that *YA*/*YA* associated with high plasma MBL levels was associated with protection against the development of new pulmonary TB. This result is consistent with that of a previous study on European descendants, which reported that *HYA*/*HYA* subjects are protected against TB [10]. Interestingly, the association was found only in younger patients when TB patients were stratified by age in our study. Nongenetic factors that may directly affect MBL levels include inflammatory condition and age, and MBL levels are higher in children than in adults [3]. In contrast, no association has been found between age and MBL levels among adults in some studies [27,28], whereas others reported that serum and plasma MBL levels in healthy adults decrease with age [29,30], although the levels were not stratified by *MBL2* genotypes in those studies. We did not find a significant age-dependent difference in MBL levels in either adult patients with TB or the control group, whereas we clearly demonstrated *YA*/*YA* diplotype-dependent effects on MBL levels even in the disease state during anti-TB treatment.

The protective role played by high MBL levels only in the younger patients with TB may be related to the complicated immune balance involved in TB development, which occurs within a shorter duration after *Mtb* infection in younger patients. On the other hand, older patients may have maintained LTBI status and *Mtb* in granulomas for a longer time. Because previous studies have suggested that the interaction between MBL and pathogens enhances proinflammatory cytokine production [31–33], high MBL levels may contribute to the maintenance of *Mtb* containment. Moreover, MBL affects monocyte and dendritic cell function at high levels without forming a complex with pathogens [34–37]. Although high MBL levels may limit *Mtb* infection into LTBI status during the younger days of a patient, the decline in the immune response with aging may result in the development of TB disease. The interaction between MBL and host cells and the subsequent immunological response should be elucidated in both the young and the elderly. The effects of associated SNPs in other genetic studies of TB are also restricted in young patients with TB [13,14,38]. Although the mechanism of age-dependent association may be different among genes, studies on the association between host genetic polymorphisms and TB should carefully consider the effects of age.

It has been speculated that the lack of MBL as an opsonin may suppress *Mtb* uptake by macrophages and prevent infection. However, we did not find significantly different plasma MBL levels and the frequencies of *MBL2* genotypes between HCWs with and without LTBI. Therefore, high MBL levels may not promote the establishment of LTBI, but *YA*/*YA* may confer protection against the development of TB. This is a new concept because the relationship between the *MBL2* genotype and TB infection, as assessed by IGRA or the tuberculin skin test, has not been clearly investigated so far. A limitation of the present study is that the number of asymptomatic individuals according to the IGRA results was relatively small; therefore, larger-scale studies are necessary to elucidate the role of MBL in various stages of TB.

In addition, differences in bacterial strains should be considered as some studies have reported associations between human polymorphisms in patients with TB and particular *Mtb* strains [39]. Furthermore, the binding of MBL to *Mtb* can be different among *Mtb* strains. A nonfunctional haplotype of a population in Ghana (*LYQC*) is associated with protection against TB caused by *Mycobacterium africanum*, which was identified in 30% of their isolates [8]. The *Mtb* Euro-American lineage accounted for 65% of their isolates, and they did not find any association between *MBL2* genotype and TB caused by *Mtb*. The distribution of *Mtb* lineages is different between Africa and Asia [24], and the Beijing genotype, which was <4% in the Ghanaian study, was identified in 58% of our Vietnamese isolates [16]. In our study, *YA*/*YA* was less frequently found in both younger patients with TB caused by the Beijing genotype strain and those with TB caused by the non-Beijing genotype strain, whereas diplotypes with low or deficient MBL levels tended to be more frequently found only in younger patients with the Beijing genotype strain. Although we should be cautious in interpreting the results of subgroup analysis, we speculate that human *MBL2* nonfunctional or low-producing alleles (*B*, *C*, *D*, and *X*) originally evolved to dampen phagocyte-mediated spreading of intracellular pathogens, including the ancestral type of *Mtb* such as *M. africanum*, that may have exploited binding to MBL [40]. However, the recently expanding modern *Mtb* strains, such as the Beijing genotype strains, may have further evolved to be unaffected by MBL deficiency or to take advantage of low MBL levels. It is postulated that 'modern' *Mtb* such as Beijing strain is more pathogenic than other 'ancient' lineages, and that infection with modern *Mtb* progresses faster to active disease presumably because it elicits weaker innate immunity and poorer defense mechanism [41]. In this context, lower MBL levels may facilitate the early progression to active disease caused by the 'modern' *Mtb*. To explain the controversial results from studies on the association between *MBL2* and TB, a study design collecting both host and bacterial information is required because the outcome of TB infection and disease depends on interactions between host and pathogen genotypes [12].

In conclusion, *MBL2 YA*/*YA*, involved with high levels of plasma MBL, played a protective role against the development of TB in younger (mean age = 32) patients in Viet Nam. Further studies are required to fully elucidate the role of MBL in *Mtb* infection and TB development.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.humimm.2014.06.006.

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Association between tuberculosis recurrence and interferon-γ response during treatment

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KEYWORDS
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Summary *Objectives:* We investigated the relationship between tuberculosis recurrence and *Mycobacterium tuberculosis* antigen-stimulated interferon-gamma (IFN-γ) responses during

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Recurrence;
Interferon-γ release assay;
Cellular response

treatment.
Methods: Plasma IFN-γ levels in active pulmonary tuberculosis patients ($n = 407$) were analyzed using QuantiFERON-TB Gold In-Tube™ (QFT-IT) at 0, 2, and 7 months of the 8-month treatment received from 2007 to 2009 and the patients were followed up for another 16 months after treatment. Risk factors for recurrence were assessed using the log-rank test and Cox proportional hazard models. Random coefficient models were used to compare longitudinal patterns of IFN-γ levels between groups.
Results: QFT-IT showed positive results in 95.6%, 86.2%, and 83.5% at 0, 2, and 7 months, respectively. The antigen-stimulated IFN-γ responses varied significantly during the treatment course ($P < 0.0001$). Unexpectedly, positive-to-negative conversion of QFT-IT results between 0 and 2 months was significantly associated with earlier recurrence (adjusted hazard ratio, 5.57; 95% confidence interval, 2.28—13.57). Time-dependent changes in IFN-γ levels were significantly different between the recurrence and nonrecurrence groups ($P < 0.0001$).
Conclusions: Although the IGRA response varies individually, early response during the treatment course may provide an insight into host immune responses underlying tuberculosis recurrence.

Introduction

Tuberculosis (TB) remains a major global health problem, resulting in 8.7 million new cases and 1.4 million deaths annually, and multidrug-resistant TB occurs in approximately 3.7% new cases and 20% previously treated cases.[1] Recurrence is thus a major risk factor for multidrug-resistant TB cases[2] and increases the TB burden.[3—5] TB recurrence is defined as a second episode of active disease as a result of relapse (endogenous reactivation) or exogenous reinfection after completion of previous treatment.[6] Biomarkers are necessary for the assessment of treatment effectiveness including early recurrence.[7]

The interferon-gamma (IFN-γ) release assay (IGRA) is an immunological diagnostic test designed to detect TB infection. In this assay, IFN-γ levels produced by primed blood lymphocytes after stimulation with *Mycobacterium tuberculosis* (MTB)-specific antigens in vitro are measured. According to the assay's principle and research findings obtained from animal models, the IGRA response may be attenuated proportional to decreased bacterial antigen load as a result of successful anti-TB treatment.[8,9] However, clinical researchers argue that little correlation exists between the commercial IGRA response and bacillary burden,[10] on the basis of various tests including grade of sputum smear or presence of cavities on chest X-rays (CXRs).[11]

Although many studies have demonstrated a decrease in IFN-γ values during treatment,[12—16] others have shown inconsistent changes and increases have also been reported occasionally.[17—20] Thus, most clinicians believe that monitoring changes in the IGRA response during anti-TB treatment may have limited use in evaluating the effectiveness of treatment[21]; however studies on the relationship of the IGRA response to subsequent episodes of TB recurrence are lacking. In this study, we investigated whether longitudinal patterns of the IGRA response during the treatment period are associated with TB recurrence.

Materials and methods

Ethics statement

A written consent was obtained from each participant. In the case of minors, the parents provided the written consent. The study was approved by the ethical committees of the Ministry of Health, Vietnam and National Center for Global Health and Medicine, Japan.

Study population

In total, 506 unrelated patients aged ≥16 years with smear- and culture-positive pulmonary TB and without history of TB treatment, were consecutively recruited from July 2007 to March 2009 in Hanoi, Vietnam. The MTB culture test was performed using Löwenstein—Jensen media. MTB isolates were subjected to niacin and drug susceptibility tests for streptomycin (SM), isoniazid (INH), ethambutol, and rifampicin. Peripheral blood samples were obtained at diagnosis before initiation of anti-TB treatment (0 months, baseline) for analyzing total blood count, human immunodeficiency virus (HIV) status, and IGRA. IGRA test was repeated at 2 months immediately after the intensive treatment period and at 7 months at the final stage of the maintenance treatment period of the standard 8-month regimen of 2SHRZ/6HE, which was commonly administered during the study period in Vietnam. CXRs were obtained at the baseline and results were interpreted by two unbiased readers blinded to the IGRA results. In the present analysis, patients with multidrug-resistant TB as well as HIV coinfection were excluded.

Follow-up and definitions

During treatment, culture tests were repeated when smear tests were confirmed positive at 2, 5 or 7 months. During the 16-month post-treatment follow-up, sputum smear and culture tests were performed at 2, 4, 7, 10, and 16 months for all accessible cases.

Treatment failure was defined based on the WHO Global Tuberculosis Report in 2012,[1] when the smear and culture were positive at $\geq$5 months or when the smear was positive but culture was not performed, clinical and/or CXR findings indicated failure, and category switched to category II of anti-TB treatment.

Recurrence was defined when patients were cured after treatment, and then suffered from the second TB episode. The second episode was bacteriologically confirmed if the sputum culture was positive at the time of recurrence or the smear was positive or the culture revealed $<$5 colonies, clinical and/or CXR findings indicated recurrence, and category switched to category II anti-TB treatment.

Interferon-gamma release assay

An enzyme-linked immunosorbent assay-based IGRA kit, QuantiFERON-TB Gold In-Tube™ (QFT-IT; Cellestis, Victoria, Australia), was used for analysis. The guidelines for algorithm and software (QuantiFERON-TB Gold Analysis Software, version 2.50; Cellestis) provided by the manufacturer were followed for the interpretation of results. The testing procedure was carefully monitored as described earlier,[22] and test quality control was performed during each run according to the manufacturer's instructions. When IFN-γ values of negative control "Nil" and positive control "Mitogen-Nil" fell within the appropriate range, the QFT result was assessed as positive when IFN-γ value of "TBAg-Nil" was above the cutoff value (0.35 IU/ml) and negative when the value was below the cutoff value. A positive-to-negative change of QFT results was designated as "negative conversion" in this study.

Measurement of cytokines and chemokines in QuantiFERON-TB Gold In-Tube™ samples

Cytokines and chemokines released in QFT-IT plasma supernatants were collected before treatment and at 2 months and 7 months after the initiation of treatment, from 10 randomly selected recurrence patients and 10 age- and sex-matched nonrecurrence patients were measured using Bio-Plex multiplex system with a 27-plex cytokine-bead kit (Bio-Plex Pro Human Cytokine 27-plex Assay; Bio-Rad Laboratories Hercules, CA). Only values within the asymptotic range were calculated using the standard curve for statistical analysis.

Statistical analysis

Chi square or log-rank tests were used to compare the incidence of recurrence (events) between groups. Influence of time course on the proportion of IGRA-positive results was assessed using generalized estimating equations. Wilcoxon's rank-sum test was used to compare nonparametric distributions between groups. A logistic regression model was used to investigate risk factors involved in treatment failure. The log-rank test of equality across strata and Cox models after testing the proportional hazard assumption were used to assess risk factors for recurrence. The random coefficient model was used to assess influence of time course on the IGRA response and the post-estimation Wald test was used to compare the longitudinal patterns of the response between recurrence and nonrecurrence groups. Bonferroni's correction was applied to correct multiple comparisons. When the IFN-γ value was greater than 10.00 IU/ml, statistical analysis was performed in both two conditions, using a truncated value (10.00 IU/ml) or a value based on extrapolation. Truncated values are presented in parenthesis along with those based on extrapolation when appropriate. The statistical results confirmed that all significant differences found here were demonstrated in both conditions. *P* values of $<$0.05 were considered statistically significant unless otherwise specified. Statistical analysis was performed using Stata version 11 (StataCorp, College Station, TX).

Results

Characteristics of the study population

The characteristics of 506 patients recruited have been reported elsewhere.[23] In the present study, we analyzed 407 patients who were enrolled in the directly observed treatment, short-course (DOTS) program at various study sites and did not have multidrug-resistant TB or HIV coinfection at the time of initial diagnosis. Adherence to anti-TB therapy was supervised by the healthcare staff, in cooperation with the patients' family members under the DOTS strategy of the national TB control program. Out of these

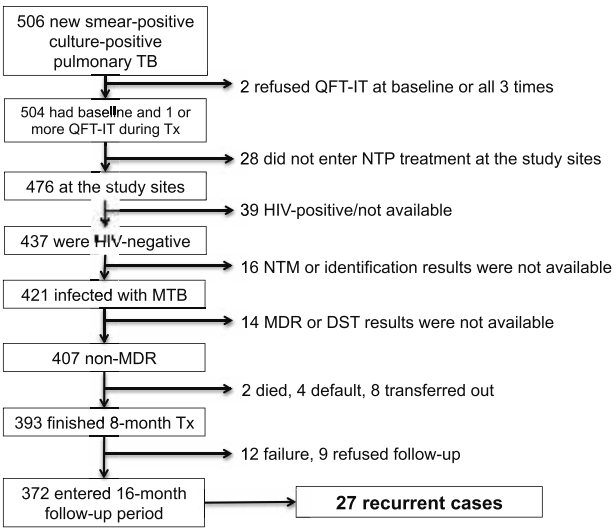

Figure 1 Study flow. TB: tuberculosis; QFT-IT: QuantiFERON-TB Gold In-Tube; NTP: National tuberculosis program; NTM: nontuberculous mycobacterium; MDR: multi-drug resistance; DST: drug sensitivity test; MTB: *Mycobacterium tuberculosis*; Tx: treatment.

Table 1 Patterns of qualitative QFT-IT results during the treatment course ($n = 407$).

QFT-IT pattern[a]		*n*	% (95% CI)
Positive-to-Positive-to-Positive	(PPP)	265	65.1 (60.3—69.7)
Positive-to-Positive-to-Negative	(PPN)	27	6.6 (4.4—9.5)
Positive-to-Positive-to-others	(PP_)	40	9.8 (7.1—13.1)
Positive-to-Negative-to-Positive	(PNP)	14	3.4 (1.9—5.7)
Positive-to-Negative-to-Negative	(PNN)	12	2.9 (1.5—5.1)
Positive-to-Negative-to-others	(PN_)	5	1.2 (0.4—2.8)
Negative-to-Negative-to-Negative	(NNN)	8	1.9 (0.9—3.8)
Others		36	8.8 (6.3—12.0)

QFT-IT: QuantiFERON TB-Gold In-tube; TB: tuberculosis; 95% CI: 95% confidence interval.
[a] QFT-IT was performed three times: before treatment, two months, and seven months after starting anti-tuberculosis treatment.

407 patients, 393 completed the 8-month standard treatment course; 381 (97.0%) were cured and 12 (3.0%) did not show treatment response (12/393). Among the cured patients, 372 (97.6%) entered the 16-month post-treatment follow-up (Fig. 1).

The median age of the 372 follow-up patients was 39.7 years (Interquartile range or IQR, 29.0—50.1); 77.7% (289/372) were male, and 238 (64.0%) were current or ex-smokers. Of the MTB isolates tested, 22.8% (85/372) displayed INH resistance with or without SM resistance, and 66.7% (248/372) were sensitive to all 4 major anti-TB drugs tested (data not shown). During the follow-up period, 27 patients (7.3%) showed recurrence.

QuantiFERON-TB Gold In-Tube™ results during treatment period

QFT-IT results were positive in 95.6% (389/407), 86.2% (337/391), and 83.5% (294/352) of the patients tested at 0, 2, and 7 months, respectively, after treatment onset. The proportion of positive IGRA responses varied significantly during the treatment course ($P < 0.0001$). The proportion of negative conversion (positive-to-negative; PN) between 0 and 2 months, 0 and 7 months, and 2 and 7 months were 7.9% (31/391), 12.2% (43/352), and 8.4% (29/347), respectively. The patterns of QFT-IT results as measured at the three time points during the course of the treatment period are shown in Table 1.

QuantiFERON-TB Gold In-Tube™ interferon-gamma values during treatment

The median values of IFN-γ, "TBAg-Nil" at 0, 2, and 7 months were 7.33 [IQR 2.53—14.53 (10.00)], 3.22 (1.03—9.54), and 2.54 (0.77—7.80) IU/ml, respectively. IFN-γ values significantly varied during the treatment course ($P < 0.0001$).

QuantiFERON-TB Gold In-Tube™ results and recurrence

The overall proportion of recurrence was significantly higher in the PN (between 0 and 2 months) group than in the positive-to-positive (PP) group [7/27 (25.9%) vs. 18/311 (5.8%), $P = 0.0001$] (Table 2). The 1-year recurrence rate was also significantly higher in the PN (between 0 and 2 months) group than in the PP group {25.9% [95% confidence interval (CI), 13.3—46.8] vs. 5.5% [95% CI, 3.4—8.7]}. The log-rank test

Table 2 Proportion of treatment failure and recurrence in TB patients showing positive-to-positive and positive-to-negative patterns of QFT-IT results.

Patterns of QFT-IT results	Treatment failure *n/N* (%)	*P* value[a]	Recurrence *n/N* (%)	*P* value[a]
Between month 0 and month 2				
Positive-to-Positive	8/325 (2.5)	0.203	18/311 (5.8)	0.0001
Positive-to-Negative	2/30 (6.7)		7/27 (25.9)	
Between month 0 and month 7				
Positive-to-Positive			19/285 (6.7)	0.222
Positive-to-Negative			5/43 (11.6)	
Between month 2 and month 7				
Positive-to-Positive			15/264 (5.7)	>0.999
Positive-to-Negative			1/29 (3.5)	

TB: tuberculosis; QFT-IT: QuantiFERON-TB Gold In-Tube.
[a] By Chi square or Fisher's exact test; comparisons were made between the two groups with different patterns of QFT-IT results; Positive-to-Positive and Positive-to-Negative.

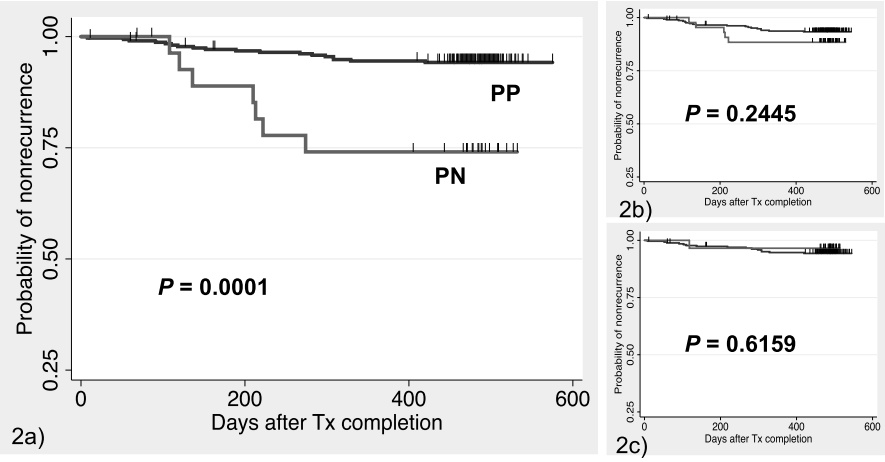

Figure 2 Kaplan–Meier plots stratified by the conversion of QFT-IT results between 0 and 2 months (2a), 0 and 7 months (2b), and 2 and 7 months (2c). QFT-IT: QuantiFERON-TB Gold In-Tube; Tx: treatment; Blue line: positive-to-positive (PP) QFT-IT results. Red line: positive-to-negative (PN) QFT-IT results. The *P* values were obtained by the log-rank test.

confirmed the difference between the two groups ($P = 0.0001$; Fig. 2a), whereas the conversion of QFT-IT results between 0 and 7 months and between 2 and 7 months did not affect recurrence ($P = 0.2445$ and $P = 0.6159$, Fig. 2b and c, respectively). Among the 27 recurrence cases, MTB isolates were sensitive to all drugs tested in 14 cases (51.9%). INH resistance with or without SM resistance was seen in 8 cases (29.6%). This percentage was slightly higher than that of the nonrecurrence group (77/345 or 22.3%), but the difference was not statistically significant ($P = 0.189$, data not shown). The proportion of this drug resistance was also not different between groups with and without the negative conversion (7/31 or 22.6% vs. 83/332 or 25.0%, $P = 0.919$) (data not shown). Using the Cox proportional hazard model, the association between recurrence and the negative conversion of QFT-IT results between 0 and 2 months remained significant (hazard ratio, 5.57; 95% CI, 2.28–13.57) after adjusting for BMI at baseline, smear results at 2 months, drug resistance, and smoking status in the final model (Table 3).

QuantiFERON-TB Gold In-Tube™ interferon-gamma values and recurrence

We further assessed possible changes in the actual IFN-γ values using a random coefficient model with log-transformed IFN-γ values of "TBAg-Nil" set as an outcome variable and time of testing, recurrence status, and the

Table 3 Multivariate analysis using Cox proportional hazard model[a] to assess risk factors for recurrence ($n = 372$).

	Proportion (%)	Hazard ratio	95% CI
QFT-IT status at baseline to 2 months after starting treatment			
Positive-to-Positive	18/311 (5.8)	Reference	–
Positive-to-Negative	7/27 (25.9)	5.57	2.28–13.57
BMI		0.86	0.71–1.04
Result of sputum smear at 2 months after starting treatment			
Negative	22/326 (6.8)	Reference	–
Positive	5/46 (10.9)	2.28	0.84–6.16
Drug resistance profile			
Sensitive to all 4 drugs tested[b]	14/248 (5.7)	Reference	–
INH resistance (± SM resistance)	8/85 (9.4)	1.66	0.65–4.19
Other resistant patterns	5/39 (12.8)	2.77	0.96–8.06
Smoking status			
No	8/134 (6.0)	Reference	–
Yes[c]	19/238 (8.0)	1.48	0.61–3.61

95% CI: 95% confidence interval; QFT-IT: QuantiFERON-TB Gold In-Tube; BMI: body mass index; INH: isoniazid; SM: streptomycin.
[a] Initial model included BMI, sex, age, status of QFT-IT results at baseline to 2 months after starting treatment, presence of cavity or extension of infiltrate on chest radiograph, the results of smear testing at 2 months, patterns of drug resistance, and smoking status. Variables showing $P > 0.2$ were removed from the final model.
[b] The drugs INH, SM, rifampicin, and ethambutol were tested.
[c] Current or ex-smokers.

Table 4 Analysis of time-dependent change of interferon-γ values during treatment period using random coefficient model.

	Coefficient	*P* value	95% CI
TBAg−Nil (log-transformed values) as outcome variable[a]			
Month 2	−0.64	<0.001	−0.74 to −0.54
Month 7	−0.96	<0.001	−1.09 to −0.82
Recurrence	−0.17	0.535	−0.70 to 0.36
Interaction term between 2 months and recurrence	−0.83	<0.001	−1.20 to −0.46
Interaction term between 7 months and recurrence	−0.17	0.513	−0.67 to 0.33
Constant	1.73	<0.001	1.58 to 1.87
Mitogen−Nil (log-transformed values) as outcome variable[a]			
Month 2	0.43	<0.001	0.26 to 0.59
Month 7	1.05	<0.001	0.88 to 1.22
Recurrence	−0.04	0.902	−0.61 to 0.54
Interaction term between 2 months and recurrence	0.22	0.465	−0.37 to 0.81
Interaction term between 7 months and recurrence	−0.04	0.904	−0.64 to 0.57
Constant	1.12	<0.001	0.96 to 1.28

95% CI: 95% confident interval; TBAg−Nil: tuberculosis-specific antigen values minus Nil values; Mitogen−Nil: mitogen values minus Nil values.

[a] 0 month and nonrecurrence group are reference categories.

interaction between the two as independent variables. IFN-γ levels differed significantly with time between the recurrence and nonrecurrence groups according to the post-estimation Wald test ($P < 0.0001$) (Table 4). Fig. 3 shows the linear prediction lines of nonrecurrence and recurrence groups, being overlaid on the basis of individual changes in IFN-γ values.

Statistical significance in the overall difference in QFT-IT IFN-γ values between the two groups prompted us to characterize further estimates. Among the three time points measured, IFN-γ values at 2 months were significantly lower in the recurrence group than in nonrecurrence group {1.36 [IQR, 0.25−3.15] vs. 3.82 [1.12−10.51 (10.00)] IU/ml, $P = 0.003$}, whereas IFN-γ values at 0 months were not significantly different between the recurrence and nonrecurrence groups ($P = 0.1467$). In addition, magnitudes of IFN-γ level changes measured at two consecutive points of time were divided equally into three levels for comparing recurrence time. This indicated that increase of IFN-γ values between 2 and 7 months was significantly associated

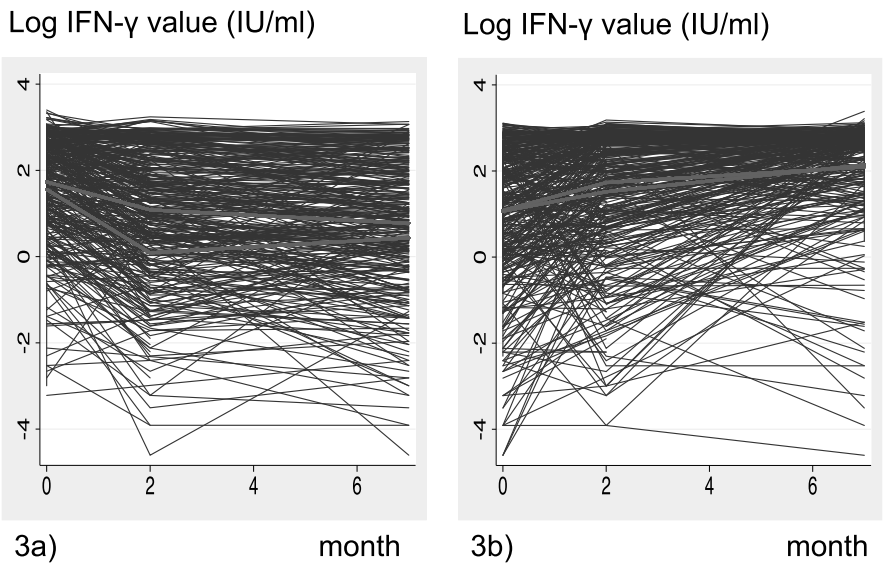

Figure 3 Linear prediction of transition patterns of interferon-γ that responded to TB-specific antigens (3a) and to mitogen (3b) among recurrence and nonrecurrence cases. TB: tuberculosis; IFN-γ: interferon-γ; Blue line: individual IFN-γ pattern; Upper red line: linear prediction line of nonrecurrence group; Lower red line: linear prediction line of recurrence group.

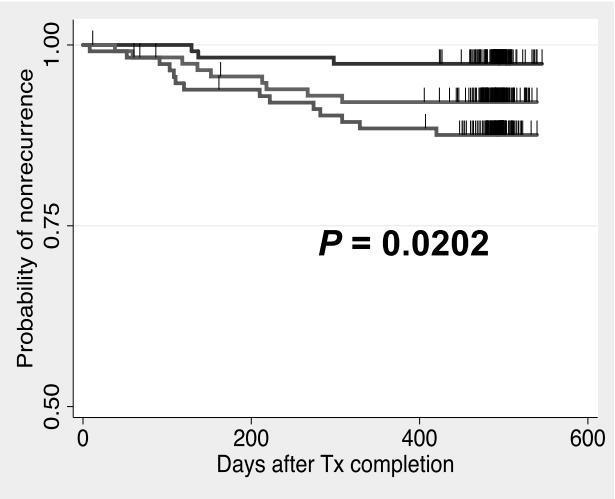

Figure 4 Kaplan–Meier plots stratified by the magnitude of increase in interferon-γ values that responded to TB-specific antigens. The magnitude of increase in interferon-γ values between 2 and 7 months was divided equally into three levels: small (blue line), medium (red line) and large (green line); TB: tuberculosis; Tx: treatment; The *P* value was obtained by the log-rank test.

with TB recurrence ($P = 0.0202$). Kaplan–Meier plots are shown in Fig. 4.

QuantiFERON-TB Gold In-Tube™ and treatment failure

The proportion of failure was slightly higher in the PN group (between 0 and 2 months) than in the PP group (Table 2), although no significant association was found even after we ran the logistic regression model with treatment failure as an outcome variable, and the status of negative conversion and result of smear testing at 2 months both as independent variables (data not shown). However, similar to the results observed between IFN-γ patterns and recurrence, the IFN-γ values of "TBAg-Nil" at 2 months were significantly lower in the failure group than in the cure group [median = 1.04, (IQR = 0.21–3.44) vs. 3.46 (1.03–9.82) IU/ml, $P = 0.0285$].

Cytokines and chemokines in QuantiFERON-TB Gold In-Tube™ plasma supernatants after stimulation with tuberculosis-specific antigens

Among the 27 cytokines and chemokines tested, IL-2, IL-1RA, IP-10, and IFN-γ levels were increased after TB-specific antigen stimulation, and the levels were significantly different compared with unstimulated control levels (data not shown). IL-2, IP-10, and IFN-γ levels tended to be lower in the recurrence group than in the nonrecurrence group (Table 5). Difference in IP-10 and IFN-γ levels at 2 months remained significant even after Bonferroni's correction. IL-10 levels were not different between the conditions (Table 5).

Discussion

In our study, >80% patients had a positive IGRA response until treatment completion, although TB-antigen-stimulated IFN-γ values gradually decreased with time. Interestingly, negative-conversion of the IGRA response after 2 months of treatment was significantly associated with early TB recurrence, and longitudinal patterns of the IGRA response during the treatment course were different between the recurrence and nonrecurrence groups.

According to most of the previous studies, the proportions showing positive IGRA responses before, during, and after anti-TB treatment tend to decrease in a time-dependent manner, but are largely variable.[12–15] High proportions of positivity before and during the treatment period in our study may have resulted from strong TB-antigen-specific IFN-γ response before treatment (median IFN-γ levels = 7.33 IU/ml), presumably because of high bacillary burden in immunocompetent individuals. IFN-γ levels are known to not easily decrease below the cutoff level in such cases.[24,25] Frequent exposure to MTB is one of the reasons for relatively high IFN-γ values during treatment course.[26]

Nevertheless, our study showed that IFN-γ values varied and gradually decreased with time. This finding is consistent with earlier reports,[12–15] but varies from other results in which IFN-γ values did not change remarkably[17] or increased.[19] In the Indian study cohort,[17] a large proportion of the subjects were hospitalized as compared with our study (80.0% vs. 22.1%), and the patients may have suffered from more severe disease than those in the present study. In such cases, recovery or elevation of IFN-γ levels may be observed after starting the effective treatment, although the bacterial antigen load may decrease. Such a paradoxical response may be often observed after several days of blood-cell incubation, to allow proliferation of IFN-γ-producing cells, as reported previously.[27]

Negative conversion of the IGRA response after 2 months of treatment was significantly associated with early recurrence in our cohort, even after adjustment for possible confounding factors. This was different from our expectation; no studies have attempted a possible association between actual recurrence and the IGRA response during treatment. TB antigen-specific IFN-γ levels observed at 2 months were also significantly lower in the recurrence group than in the nonrecurrence group. Pretreatment IFN-γ levels were slightly lower in the recurrence group (median = 6.03 IU/ml) than in the nonrecurrence group (median = 7.89), but were considerably higher than the cutoff value. Therefore, frequent negative conversion at 2 months in the recurrence group cannot be attributed to a simple fluctuation of IFN-γ levels around the cutoff value. As a possible confounder, the proportion of INH-resistance was not significantly associated with recurrence or negative conversion in our study, indicating that 2SHRZ/6HE, a previous standard treatment regimen during the study period in this area, did not affect our main findings.

In general, TB recurrence tends to occur when the initial disease course is severe and prolonged.[28] Peripheral blood cells may not sufficiently respond to TB-specific antigens in such a disease state. Notably, suppression of IFN-γ pro-

Table 5 Levels of IL-2, IL-1RA, IP-10, IFN-γ, and IL-10 in QFT-IT supernatants with and without TB-antigen stimulation in nonrecurrence and recurrence groups.

	No stimulation (Nil tubes)[a]		*P* value[b]	Stimulated with TB-antigens (TBAg tubes)[a]		*P* value[b]
	Nonrecurrence $n = 10$	Recurrence $n = 10$		Nonrecurrence $n = 10$	Recurrence $n = 10$	
IL-2						
Month 0	9.55 (4.99–10.00)	9.67 (6.45–11.96)	0.6480	119.59 (71.33–301.01)	87.65 (48.07–147.28)	0.1509
Month 2	9.42 (2.77–9.92)	9.42 (3.67–10.00)	0.7606	108.22 (67.52–199.84)	42.55 (34.23–90.57)	0.0588
Month 7	9.42 (2.59–9.92)	9.67 (2.59–10.00)	0.5656	80.86 (43.25–182.72)	40.26 (20.25–84.47)	0.1124
IL-1RA						
Month 0	383.02 (211.01–590.51)	506.14 (316.64–593.18)	0.4057	783.99 (528.86–1177.69)	920.99 (648.56–1065.19)	0.7624
Month 2	184.74 (115.41–298.36)	267.38 (205.48–469.55)	0.0821	455.26 (220.52–758.45)	406.91 (325.55–593.61)	>0.9999
Month 7	144.68 (120.22–158.43)	252.82 (197.18–306.53)	0.0156	360.04 (130.28–528.86)	328.62 (255.49–690.86)	0.4497
IP-10						
Month 0	6465.02 (3950.00–9531.21)	7291.13 (4614.94–13,068.25)	0.4497	136,362.20 (90,912.67–151,897.60)	86,495.93 (53,311.35–120,082.80)	0.0638
Month 2	5511.37 (2435.73–10,045.31)	7256.33 (5237.79–9094.84)	0.4497	128,971.10 (60,384.23–151,897.60)	38,334.16 (17,740.69–53,528.71)	**0.0072**
Month 7	3823.80 (1085.59–5388.28)	7182.96 (3012.90–14,986.52)	0.1736	106,655.30 (28,361.69–151,897.60)	32,663.32 (17,160.10–102,471.50)	0.1038
IFN-γ						
Month 0	126.01 (99.82–211.41)	124.17 (98.70–154.42)	0.9698	784.28 (387.67–1657.85)	393.75 (278.23–600.60)	0.0696
Month 2	81.33 (66.62–102.40)	102.40 (67.86–123.09)	0.2550	391.68 (217.13–1393.35)	157.00 (136.92–174.37)	**0.0041**
Month 7	86.21 (56.12–94.53)	98.34 (74.93–139.49)	0.1730	266.15 (116.70–590.04)	153.38 (113.18–316.82)	0.5453
IL-10						
Month 0	6.40 (6.12–7.97)	6.82 (6.21–12.49)	0.4267	6.12 (5.86–6.21)	7.13 (6.21–11.84)	0.0443
Month 2	6.12 (6.07–6.21)	6.21 (6.07–12.09)	0.2215	6.07 (5.90–6.12)	6.17 (6.07–6.66)	0.1457
Month 7	6.17 (6.07–6.25)	6.17 (5.86–6.59)	0.9393	6.07 (5.86–6.12)	6.21 (5.86–6.78)	0.1264

QFT-IT: QuantiFERON TB-gold In-tube; TB: tuberculosis; TbAg: tuberculosis-specific antigens; NS: non-significant.

[a] Values (pg/ml) are expressed in median (interquartile range).

[b] Compared between non-recurrent and recurrent groups. The *P* values were obtained by Wilcoxon's rank-sum tests; values in bold and underlined are those remained significant after Bonferroni's correction.

duction from CD4+ T-cells in response to certain TB-specific peptides has been observed in patients with severe pulmonary TB.[29] In such a condition, inhibitory receptors and soluble factors that induce T-cell anergy, such as CTLA-4 and IL-10,[30] contribution of regulatory T (Treg)-cells,[31–34] and compartmentalization of TB antigen-specific T-cells[35] may have played a role in low IFN-γ levels. In our study, however, the extent of pulmonary TB lesions, an indicator of TB severity (data not shown), and IL-10 production at 2 months, a marker of Treg activity, were not different between the two groups. Furthermore, according to the abovementioned mechanisms, IFN-γ levels should have been suppressed before treatment and recovered during effective treatment, contrary to the pattern observed in this study. Difference caused by nonspecific immune suppression that may occur in malnutrition or other states is also unlikely because the amount of nonspecific mitogen-induced IGRA response was not significantly different between the two groups (Fig. 3b).

Although our study provided no direct evidence, the impairment of T-cell memory function at the convalescent stage may have caused both the negative conversion and the low IFN-γ values seen in the IGRA results at 2 months in the recurrence group. Several lines of evidence have demonstrated that antigen-specific IFN-γ-only-secreting effector T cells are predominant in untreated active TB disease and also indicate that when the antigen load decreases after starting treatment, dual IFN-γ/IL-2- or single IL-2-secreting T-cells with more memory-cell characters become more predominant.[27,36–40] In the recurrence group, it is possible that dual IFN-γ/IL-2-secreting or polyfunctional T-cells have failed to expand for unknown reasons, whereas IFN-γ-only-secreting cells have continuously decreased, resulting in a significant reduction in overall IFN-γ production. Indeed, IL-2 induction in QFT-IT tended to be lower parallel with the lower IFN-γ response at 2 months in the recurrence group than in the nonrecurrence group. Further immunological studies on lymphocyte subpopulations would be necessary to elucidate the underlying mechanism.

In addition to the low IFN-γ values at 2 months, the increase in IFN-γ values between 2 and 7 months was also associated with early TB recurrence. A previous study[14] revealed a minor and insignificant increase in IFN-γ levels at 6 months after treatment completion in subjects having recurrence risk. It is well known that the IGRA response is higher in active TB than in latent TB infection despite a large overlap,[41–43] and the cytokine-producing capacity of MTB-specific CD4 and CD8 T-cells is associated with increased bacillary burden.[27,38] Collectively, a slight increase in IGRA responses between 2 and 7 months in the recurrence group may indicate significantly increased bacillary burden in the subclinical stage before recurrence.

Additionally, in the treatment failure group, the tendency for decreased IFN-γ levels at 2 months may not be explained by only change in bacillary burden, because the burden should be considerably higher in the failure group. The similarity in IFN-γ level patterns between recurrence and failure may suggest a common underlying mechanism, possibly the impairment of T-cell function, although the statistical power of our study was not strong enough to analyze this with regard to treatment failure.

IP-10 is a small chemokine expressed by antigen-presenting cells and it is induced by IFN-γ.[44] IP-10-based tests are comparable to the IGRA response[44] in different groups of TB-related subjects, including HIV-uninfected and infected subjects evaluated at the time of TB diagnosis or over time.[45,46] However, our study results are not conclusive about the better indicator among the two.

We did not distinguish reinfection from relapse in this study. However, we assumed that relapse cases are predominant because recurrence occurred during the short follow-up period (16 months). It should be emphasized that our findings cannot be used for the prediction of recurrence, because a variety of individual variations in longitudinal patterns of the IGRA response were observed. Nevertheless, our findings provide additional insights on the clinical relevance of IGRA in TB management. Negative conversion of the IGRA response after 2 months of treatment was not a good sign though it is widely believed to indicate clearance of infection.

The strength of the present study lies in the high proportion of patients completing treatment and active follow-up. However, this study has a few limitations. First, the last IGRA was performed at 7 months of treatment. The magnitude of change in IFN-γ values in the recurrence group may have been larger if the evaluation was performed at a later stage. Second, in our settings, we were unable to study the host immune response in detail so as to elucidate the underlying mechanism.[27,47] Third, diabetes, one of the possible confounding factors for TB recurrence, was not actively screened in our study protocol. However, the frequency of diabetes based on a questionnaire-based interview was relatively low (4.6%) in the study population and nonexistent (0%) in the recurrence group. Therefore, we did not include this factor in the multivariate analysis. Nevertheless, our data showed a negative association between IGRA response and recurrence, which may prompt future studies in this field.

In conclusion, this study showed that the patterns of IGRA responses to TB-specific antigens during treatment differ according to recurrence status and thus may provide insights into the immunological background prior to TB reactivation, a major question in this field.

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Original Article

Salmonella Meningitis: a Report from National Hue Central Hospital, Vietnam

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SUMMARY: Four Vietnamese infants who survived infection with *Salmonella* meningitis are reported. A female infant who experienced relapse recovered without complications and another had neurological sequellae. The remaining 2 infants survived without complications. The initial treatment was chloramphenicol and ceftriaxone, whereas a change of antibiotics to imipenem and fluoroquinolone was required for 2 infants. Fluoroquinolone may be a treatment option in patients with *Salmonella* meningitis who experience complications even though the drug is contraindicated for the pediatric age group.

INTRODUCTION

Four cases of *Salmonella* meningitis, considered to have a poor prognosis in neonates or infants, are reported from National Hue Central Hospital, Vietnam. National Hue Central Hospital treats more than 10,000 children who are admitted annually. The hospital is located in Hue, which is a city in central Vietnam with a population of more than one million people. All 4 infants were Vietnamese and less than 1-year-old. Their ages ranged from 32 days to 6.5 months. The choice of antibiotic used to treat *Salmonella* meningitis is important because several antibiotics routinely used to treat bacterial meningitis of other causes are reportedly ineffective as the initial treatment. The 2 initial cases were successfully treated with cephalosporin and chloramphenicol (CP), whereas the other 2 cases required therapy with imipenem (IPM) and fluoroquinolone. The average duration of antibiotic therapy was 7 weeks. One patient suffered recurrence of meningitis after 4 weeks of treatment, and the second patient experienced neurologic complications with bilateral ventricular dilatation and subdural effusion. Although a third generation cephalosporin and CP were the first line treatment for *Salmonella* meningitis until recently, IPM and fluoroquinolone should also be considered when gram-negative rods are detected in the cerebrospinal fluid (CSF). Although the indication for fluoroquinolone in children is controversial, this drug should be considered as an effective choice for this type of pediatric meningitis.

MATERIALS AND METHODS

A retrospective analysis via chart review was performed for 4 children who were treated for *Salmonella* meningitis between 2003 and 2008 at National Hue Central Hospital in Hue, Vietnam. *Salmonella* meningitis was defined as purulent meningitis when *Salmonella* sp. was cultured from CSF at the onset of the disease. Clinical review included patient history, symptoms, signs, laboratory data, treatment, course, and outcome. Laboratory data included an examination of blood, CSF, and bacterial culture. The isolated bacteria were examined for sensitivity to the following antibiotics: ampicillin (ABPC), cefazolin (CEZ), cefotaxime (CTX), ceftriaxone (CTRX), IPM, gentamicin (GM), CP, norfloxacin (NFLX), ofloxacin (OFLX), and ciprofloxacin (CPFX).

RESULTS

Among 187 cases of purulent meningitis treated at Hue Central Hospital from 2003 to 2008, 4 were caused by *Salmonella* sp. The clinical courses of the 4 cases were as follows.

Case 1: A 3.5-month-old female was admitted to Hue Central Hospital in November 2003 following 1 week of fatigue, fever, and diarrhea. On admission, her appearance was poor because of unconsciousness, convulsions, and a temperature of 40°C. She exhibited a decerebrate posture and weak papillary light reaction without anisocoria. Bulging fontanelle, a stiff neck, and Kernig's sign were absent. There were no respiratory or circulatory abnormalities. She was born at term after an uncomplicated pregnancy with a weight of 3,100 g, and she had been well until disease onset. Her parents were healthy without gastrointestinal symptoms. Her CSF was turbid because of pleocytosis, and gram-negative bacilli were present. CSF culture yielded *Salmonella enterica* Claibornei. She was discharged in good health after 4 weeks of treatment with CTRX (100 mg/kg/day) plus CP (100 mg/kg/day), but was readmitted because of recurrence of *Salmonella* meningitis 2 weeks later. She recovered following an additional 8-week course of treatment with the same antibiotic combination.

Case 2: A 32-day-old female was admitted in September 2004. The child displayed refusal to feed with lethargy and a weak cry beginning 1 day before admission. She had high-grade persisting fever, bulging anterior fontanelle, and convulsions. A motor system ex-

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Table 1. Clinical and laboratory features of 4 cases

Case	1	2	3	4
Gender	female	female	female	female
Age (Mo)	3.5	32 days	6.5	5
Admission date	10/Nov/2003	12/Sept/2004	08/Aug/2008	08/Aug/2008
Admission[1)]	7 days	1 day	1 day	1 day
Continuous fever (≥38.5°C)	+	+	+	+
Vomiting	—	—	+	+
Blood examination				
Hb (g/dl)	8.6	9.2	8.4	8.6
Leukocytes ($\times 10^9$/l)	12.5	3.8	6.2	13.7
Neutrophils (%)	56	47.3	56.6	77.3
CRP (mg/l)	51.4	124.8	176.2	223.9
Glucose (mmol/l)	6.2	6.7	5.6	7.7
Na^+ (mmol/l)	130	132	130	131
Blood culture	—	—	—	—
CSF examination				
WBC/mm^3	2,500	2,160	620	1,440
Neutrophils (%)	90	95	95	85
Gram stain	GNR	GNR	GNR	GNR
Glucose (mmol/l)	0.2	0.2	0.8	1.0
Protein (g/l)	1.71	10.75	1.56	1.50
Culture	*Salmonella* Claibornei	*Salmonella arizonae*	*Salmonella* sp.	*Salmonella* Paratyphi B
Antibiogram[2)]	Sensitive to all antibiotics	Sensitive to all antibiotics	Sensitive to all antibiotics	Sensitive to all antibiotics
Stool examination				
Stool culture	—	—	—	—

1): disease day after onset.
2): Sensitivity was tested for ABPC, CEZ, CTX, CTRX, IPM, GM, CP, NFLX, OFLX, and CPFX.
Mo, months; GNR, gram-negative rods; ABPC, ampicillin; CEZ, cefazolin; CTX, cefotaxime; CTRX, ceftriaxone; IPM, imipenem; GM, gentamicin; CP, chloramphenicol; NFLX, norfloxacin; OFLX, ofloxacin; CPFX, ciprofloxacin.

Table 2. Clinical courses

Case	1	2	3	4
Initial antibiotics	CTRX + CP	CTRX + CP	CTRX + CP	CTRX + CP
2nd antibiotics			IPM + CPFX	IPM + CPFX
Stable general state[1)]	13 days	11 days	13 days	12 days
1st treatment duration	4 weeks	7 weeks	8 weeks	6 weeks
Time of relapse after treatment	2 weeks	—	—	—
2nd treatment duration	8 weeks	—	—	—
Post-treatment fontanel echography	—	—	Dilated lateral ventricles, subdural fluid collection	—

1): hospital days after admission.

amination revealed increased tone in the extremities and hyperreflexia with clonus. She was delivered normally near term weighing 2,200 g, and she cried immediately after birth. No abnormalities were observed during the neonatal period. Her parents were healthy without infectious symptoms. CSF was turbid because of pleocytosis, displaying gram-negative bacilli, and *Salmonella enterica* subsp. *arizonae* was cultured. She was administered CTRX (100 mg/kg/day) and CP (100 mg/kg/day) and discharged in good health after 7 weeks of treatment.

Case 3: A 6.5-month-old female was admitted in August 2008 due to high fever and convulsions with loss of consciousness. Her medical history was unremarkable. She was delivered at term weighing 3,300 g. She had been febrile, with vomiting and diarrhea, for 4 days before admission. On physical examination, she was febrile, lethargic, and mildly dehydrated with brisk lower limb reflexes. CSF was turbid because of pleocytosis, displaying gram-negative bacilli, and cultures yielded *Salmonella* sp. She was discharged in good health after an 8-week course of treatment with IPM (50 mg/kg/day) and CPFX (30 mg/kg/day) following the initial treatment with CTRX and CP. Subdural effusion and hydrocephalus during her clinical course resulted in mild-to-moderate neurological deterioration.

Case 4: A 5 month-old female was admitted in September 2008 due to high fever, restlessness, convulsions, nausea, and coughing. Her medical history included hospitalization 2 weeks earlier for acute gastroenteritis. Stool culture had yielded no *Salmonella* sp. on that occasion. She was delivered vaginally at term, after an uncomplicated pregnancy, weighing 2,600 g. Her parents and elder sister were healthy without gastrointestinal symptoms. Her CSF was turbid because of pleocytosis, exhibiting gram-negative bacilli, and culture yielded *Salmonella* Paratyphi B. She was discharged in good health after 6 weeks of treatment with IPM and CPFX

following treatment with CTRX and CP. Laboratory examinations revealed leukocytosis and pleocytosis of CSF in all cases. Widal reactions were all negative. Bacterial culture isolated *Salmonella* spp. from CSF but not from stool samples.

DISCUSSION

Bacterial meningitis is an important pediatric disease because it generally has a poor prognosis in infants and young children, but intact survival without neurological sequel can be expected if the illness is diagnosed early and treated promptly. *Salmonella* infection as a cause of bacterial meningitis is reported primarily in tropical areas (1,2), but also occasionally in industrialized countries (3–5). Most cases of *Salmonella* meningitis involve children less than 1 year of age, primarily less than 3 months old (3,4). Underlying disorders, such as human immunodeficiency virus infection, malaria, or malnutrition, may act as causative factors. The poor prognosis of *Salmonella* meningitis has been emphasized to reflect that of bacterial meningitis in general (6). The mortality rate and incidence of neurological complications because of *Salmonella* meningitis are high, especially in Africa (6,7)

The 4 patients described here had typical clinical features of *Salmonella* meningitis, and they recovered following repeated administration or a change of antibiotics. Salmonellosis is recognized as a food borne infection, and the route is believed to be the same in infants. Gastrointestinal symptoms are rare in mothers and other family members even though most cases occur in neonates or infants.

Reports on *Salmonella* surveillance in asymptomatic family members of patients are rare. However, *Salmonella* infection is suspected when there might be a carrier of *Salmonella* among family members. As the mother has the most contact with an infant, the pathogen may readily be transmitted from mother to child. There are case reports of *Salmonella* meningitis that have described the isolation of *Salmonella* from maternal breast milk (4,8). Breast milk is known to be protective against many infectious diseases. It has thus been emphasized that breastfeeding should not be stopped or reduced because of the risk of transmitting the *Salmonella* meningitis pathogen, considering that breast milk itself reduces *Salmonella* infection rates in children (9).

Gram-negative rods were identified in CSF by microscopic examination, and *Salmonella* spp. were cultured in the 4 cases described. Laboratory findings for this organism were the same as those for other causes of bacterial meningitis. Blood examination revealed leukocytosis and positive inflammatory reactions. CSF exhibited pleocytosis, decreased glucose levels, and increased protein content. Blood cultures were negative in all 4 cases.

The initial treatment was CTRX plus CP. Case 1 experienced recurrence of meningitis despite the apparent success of the initial treatment. She was retreated with the same antibiotic combination. Antibiotics were changed due to slow clinical responses in cases 3 and 4. Subdural abscess and dilated ventricle resulted in neurological delay in case 3. *Salmonella* meningitis should be considered when gram-negative rods are identified in CSF. Gram-negative rods that cause meningitis include *Escherichia coli*, and *Salmonella* spp. Until recently, disease recurrence was occasionally reported for *Salmonella* meningitis, even when patients received ABPC and CP for sufficient periods (10). CP has limited efficacy because of the increasing prevalence of resistance and undesirable side effects (7). It is necessary to administer the most appropriate treatment based on laboratory test findings.

Clinical improvement is achieved with IPM and fluoroquinolone. The use of fluoroquinolone is sometimes advocated (2,3,10), although its use is discouraged in young children because of possible adverse effects. The drug should be considered an effective choice for treating meningitis in special clinical settings.

In conclusion, *Salmonella* meningitis occurs mainly in tropical countries in infants, and it is considered to have a poor prognosis compared to other forms of bacterial meningitis. The selection of initial antibiotics is important for sequela-free survival. Combining IPM and fluoroquinolone may improve survival and neurological outcomes in complicated cases. The use of fluoroquinolone in children is controversial, but it should be considered as an effective treatment against bacterial meningitis caused by gram-negative rods.

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Conflict of interest None to declare.

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