e-ASIA Joint Research Program
Final Report

1. Project title：Evaluation of the pandemic potential of H5N1 highly pathogenic avian influenza viruses circulating in Indonesia.

2. Joint Research period：April 1, 2015 ～ March 31, 2018

3. Research Team：

■ **Japan team**（up to 6 people including the Principal Investigator）
Funding period: April 01, 2015–March 31, 2018
Total Funded Amount (in Local Currency): 30,258,900 JPY

	Name	Position	Affiliation	Role in the project
PI	Yoshihiro Kawaoka	Professor	Univ. of Tokyo	Supervisor
Collaborator	Kiyoko Iwatsuki-Horimoto	Assistant Professor	Univ. of Tokyo	Characterization of H5N1 viruses
Collaborator	Shinya Yamada	Assistant Professor	Univ. of Tokyo	Characterization of H5N1 viruses
Collaborator	Tadashi Maemura	PhD Student	Univ. of Tokyo	Characterization of H5N1 viruses
Collaborator	Takeaki Imamura	PhD Student	Univ. of Tokyo	Characterization of H5N1 viruses
Collaborator	Yukimasa Matsuzawa	PhD Student	Univ. of Tokyo	Characterization of H5N1 viruses
Total number of participants including students: 7				

■ **Indonesia team**（up to 6 people including the Principal Investigator）
Funding period: April 01, 2015–March 31, 2018
Total Funded Amount (in Local Currency):4,500,000,000 IDR

	Name	Position	Affiliation	Role in the project
PI	Chairul A. Nidom	Professor	Airlangga Univ.	Supervisor of Indonesia team
Collaborator	M. Kholik	Researcher	Airlangga Univ.	Sample collection
Collaborator	Ema Qurnianingsih	Researcher	Airlangga Univ.	Sample collection
Collaborator	Kadek Rahmawati	Researcher	Airlangga Univ.	Sample collection
Collaborator	Reviany V. Nidom	Researcher	Research Hospital of Tropical-Infectious Disease	Characterization of H5N1 viruses
Total number of participants including students: 5				

■ **US team**（up to 6 people including the Principal Investigator）
Funding period: April 01, 2015–March 31, 2018
Total Funded Amount (in Local Currency): 186,425 USD

	Name	Position	Affiliation	Role in the project
PI	Gabriele Neumann	Research	Univ. of Wisconsin-	Supervisor of US

		Professor	Madison	team
Collaborator	Masato Hatta	Research Associate Professor	Univ. of Wisconsin-Madison	Characterization of H5N1 viruses
Collaborator	Shufang Fan	Assistant Scientist	Univ. of Wisconsin-Madison	Characterization of H5N1 viruses
Collaborator	Gongxun Zhong	Postdoctoral Research Associate	Univ. of Wisconsin-Madison	Characterization of H5N1 viruses
Collaborator	Amie Eisfeld-Fenney	Associate Scientist	Univ. of Wisconsin-Madison	Characterization of H5N1 viruses
Total number of participants including students: 5				

4. Summary of the joint research

Highly pathogenic H5N1 avian influenza viruses cause outbreaks among poultry in Indonesia every year, leading to human infections. It is imperative that we understand the current situation regarding H5N1 virus circulation in Indonesia and characterize these viruses to assess their pandemic potential and to prepare for a potential H5N1 pandemic.

To this end, the Indonesian research team has strengthened its influenza surveillance systems to collect swab samples from poultry and pigs in outbreak areas. For example, they have strengthened their relationship with the local governments of West Kalimantan, Madura Island, Central Java province, and West Java province so that H5N1 viruses isolated in these areas can be promptly provided to the Indonesian team. From 2015 to 2017, the Indonesian team conducted surveillance on Java Island, Madura Island, and on Kalimantan Island with the Tokyo research team (Fig. 1). They sent 980 swab samples in 2015, 496 swab samples in 2016, and 1700 samples in 2017 to the Tokyo research team.

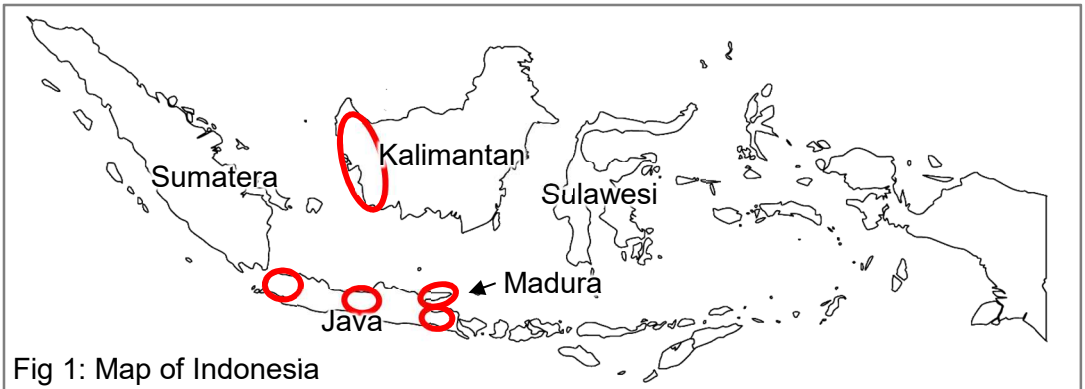

Fig 1: Map of Indonesia

The Indonesian team collected blood from Indonesian volunteers and analyzed antibody titers against H5N1 viruses. None of the elementary school children, paramedics, residents of poultry farm neighborhoods, or medical students had antibody titers against H5N1 virus; however, a few poultry farmers and live bird market workers had antibodies against H5N1 virus. These individuals may have been asymptomatically infected with H5N1 virus. This result is important to understand the clinical status of H5N1 virus in humans.

The Tokyo research team visited Indonesia and conducted surveillance with the Indonesian team in East Java, Madura Island, and West Kalimantan between 2015 and 2017. They performed virus isolation on imported swab samples by using embryonated chicken eggs in their BSL3 facility and isolated seven H5N1 viruses from the 980 samples in 2015, and 12 H5N1 viruses from the 496 swab samples collected in 2016. Phylogenetic analysis of the hemagglutinin (HA) genes showed that all 19 of these H5N1 viruses belonged to clade 2.3.2.1. Clade 2.3.2.1 viruses were thought to have been introduced into Indonesia from Vietnam in 2012. None of the isolated viruses belonged to Clade 2.1.3, which had been dominant until the introduction of

the clade 2.3.2.1 viruses. Whole genome sequencing showed that the PB2 segment (one of the virus polymerase subunits) of some of the viruses isolated in 2016 came from a different source (Fig. 2). Blast searches showed that the virus possessing the closest-related PB2 was a duck H5N2 virus isolated in Malaysia in 2004. These results suggest that clade 2.3.2.1 viruses have become predominant in Indonesia by taking the place of clade 2.1.3 viruses and reassorting with a virus of another subtype.

To assess the phenotype of the isolated viruses, we performed a minigenome assay and examined virus polymerase activity in human cells. The introduction of PB2 from the H5N2 virus reduced the polymerase activity, suggesting that the PB2 reassortment does not necessarily confer efficient replication in human cells. However, other clade 2.3.2.1 viruses showed higher polymerase activity than that of clade 2.1.3 viruses, suggesting that the recently circulating viruses have the potential to replicate efficiently in

Fig. 2: Phylogenetic tree of the PB2 gene of Indonesian H5N1 influenza viruses

humans, compared to previous viruses. Furthermore, the Tokyo research team analyzed the replicative ability in cultured cells (human respiratory epithelial cells and chicken embryo fibroblast cells) of 23 H5N1 viruses isolated between 2010 and 2016 in Indonesia (Fig. 3). They found that the growth ability of clade 2.1.3 viruses differed considerably depending on the strain tested, although all of tested clade 2.3.2.1 viruses replicated efficiently in human cells. All of the tested viruses also replicated efficiently in chicken cells. This result suggests that most of the recently circulating viruses have acquired the ability to replicate in human cells to some degree, with some viruses replicating efficiently. Analysis of the HA and neuraminidase (NA) amino acid sequences revealed that none of the isolates have acquired any mutations that are known to confer human-type receptor recognition or resistance to NA-inhibitor drugs, respectively.

The US team analyzed the pathogenicity in mice of H5N1 viruses isolated from Indonesian poultry and found that the clade 2.3.2.1 viruses showed higher pathogenicity than clade 2.1.3 viruses. PB2 reassortant viruses showed lower pathogenicity than other clade 2.3.2.1 viruses, indicating that PB2 reassortment does not confer the high virulence of H5N1 viruses. These findings suggest that the recently circulating clade 2.3.2.1 viruses possess a

phenotype of higher pathogenicity in mammals compared to that of clade 2.1.3 viruses.

To better understand the evolution of highly pathogenic H5 influenza viruses, the US team also introduced random mutations into the HA head region (where the antigenic epitopes are located) of two Indonesian H5N1 influenza viruses. As a result, they obtained so-called 'virus libraries' comprised of millions of viruses with random mutations in the HA head. These virus libraries were then incubated with sera raised against the parental wild-type viruses, resulting in the neutralization of the parental wild-type viruses. 'Escape mutants' were isolated and sequenced to identify the mutations in HA that confer antigenic escape (Fig. 4). The US team has now performed several antigenic screens and identified a number of mutants that differ antigenically from the parental wild-type viruses. This information is important to predict the evolution of Indonesian H5 influenza viruses and may aid in the selection of future candidate vaccine viruses.

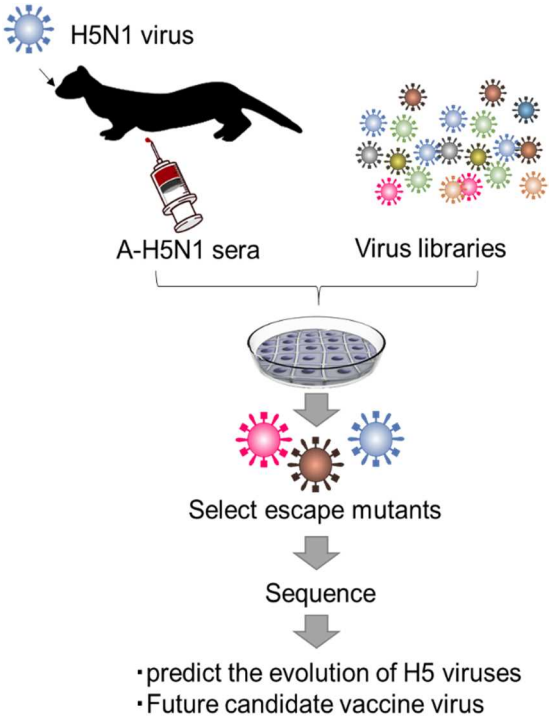

Fig 4: Isolation of escape mutants

In summary, the Indonesian team has strengthened their influenza surveillance systems and collected swab samples in Indonesia. From these samples, the Tokyo and US teams have isolated H5N1 viruses currently circulating in Indonesia and have molecularly and biologically characterized them. The information from this study improves our understanding of the current situation regarding H5N1 virus circulation in Indonesia.

5. Outputs and Anticipated Outcomes of Joint Research

5-1 Scientific achievements and implemented activities of the joint research

In this research project, we established an influenza surveillance system in Indonesia and determined the phenotype of recently circulating H5N1 viruses in Indonesia. Our findings will be helpful to deepen our understanding of the current situation with regard to H5N1 influenza virus circulation in Indonesia and will be useful to assess the pandemic potential of Indonesian H5N1 viruses to prepare for a potential H5N1 pandemic.

5-2 Synergistic effects of the international joint research

Our proposed activities were highly complementary: Surveillance activities in Indonesia were carried out by a local expert in influenza virus surveillance, Dr. Nidom. The Japanese team had long-standing expertise in receptor-binding studies and antiviral drug testing. The US team was highly experienced in studies in ferrets.
None of the teams could not carry out the proposed research autonomously, resulting in a truly complementary and highly integrated research project.

5-3 Broader impacts including contribution to society

Our research revealed that the recently circulating clade 2.3.2.1 viruses showed higher polymerase activity and higher pathogenicity in mice compared to that of previously circulating clade 2.1.3 viruses. In addition, we showed that some live bird market workers had antibodies against H5N1 virus, suggesting that these individuals may have been asymptomatically infected with H5N1 virus. These results improve out ability to understand the risk of currently circulating H5N1 viruses and also the clinical status of H5N1 virus in the human Indonesian population.

5-4 Development and sustainability of the cooperation

We succeeded in setting up an efficient system to perform a series of studies: from sample collection in the field to the phenotypical analysis of isolated influenza viruses in the lab, by building cooperative and collaborative relationships among three countries. These relationships and the system are sustainable beyond this project.

6. Future Goals and Plan of Activities after the project period

In Indonesia, as of May 2018, the total number of humans infected with H5N1 viruses reached 200; the second largest number in the world. Although no human cases were reported in 2016 or 2018, H5N1 viruses continue to cause outbreaks among poultry in many parts of Indonesia, suggesting the possibility that circulating H5N1 viruses could infect more humans. In this project, we characterized the H5N1 viruses currently circulating in Indonesia to assess their pandemic potential and to prepare for a potential H5N1 pandemic. The comprehensive characterization of H5N1 viruses currently circulating in Indonesia is essential to determine whether such viruses pose a pandemic threat. This project identified novel molecular markers of H5N1 adaptation to humans. Such information is vital for effective preparedness for and prevention of pandemics caused by H5N1 viruses not only in Indonesia but in other parts of the world.

7. Scientific Achievements and Implemented Activities (Publication, Research Exchange, Workshop, etc.)

For this item, please fill in the attached Excel file.

8. Recommendations and Comments to the Program

None

9. Others (agenda of workshop, photos of research teams, meetings, and etc.)

Photo: At the local government office of Madura (March 2016).

Lists of Achievements and Implemented Activities

1. Original Publication of Articles etc.

【Notes】
Please fill in **only the achievements of this project** by country in order of publication date. Only "published" is targeted, but please write "in press" too only for Final Report.
Please count Proceedings with peer review as original paper.
The information on this form is only disclosable. Please submit Non-disclosable information in a separate file.

1. 1 Original Publications (Articles co-authored among Research Teams)

All Authors' Names, Title, Journal Name, Volume, Edition, Page, Year of Publication	DOI Code	Publication Status	Remarks (e.g. publication in top level journals etc.)
Taro Kagaku and Jiro Kagaku、Distinct roles of MLCK and ROCK in the regulation of membrane protrusions and focal adhesion dynamics during cell m	doi: 10.1083/jcb.201506	in press	
Yamaji R, Yamada S, Le MQ, Li C, Chen H, Qurnianingsih E, Nidom CA, Ito M, Sakai-Tagawa Y, Kawaoka Y. Identification of PB2 mutations responsible for the efficient replication of H5N1 influenza viruses in human lung epithelial cells. J Virol. 2015, 89:3947-3956.	10.1128/JVI.03328-14.	published	
Ping J, Lopes T.J.S, Nidom CA, Ghedin E, Macken CA, Fitch A, Imai M, Maher EA, Neumann G, Kawaoka Y. Development of high-yield influenza A virus vaccine viruses. Nat Commun. 2015, 6:8148.	10.1038/ncomms9148.	published	
Neumann G, Kawaoka Y. Transmission of influenza A viruses. Virology. 2015, 479-480:234-246.	10.1016/j.virol.2015.03.009.	published	
Arafa AS, Yamada S, Imai M, Watanabe T, Yamayoshi S, Iwatsuki-Horimoto K, Kiso M, Sakai-Tagawa Y, Ito M, Imamura T, Nakajima N, Takahashi K, Zhao D, Oishi K, Yasuhara A, Macken CA, Zhong G, Hanson AP, Fan S, Ping J, Hatta M, Lopes TJ, Suzuki Y, El-Husseiny M, Selim A, Hagag N, Soliman M, Neumann G, Hasegawa H, Kawaoka Y. Risk assessment of recent Egyptian H5N1 influenza viruses. Sci Rep 2016, 6:38388.	10.1038/srep38388.	published	
Sakai-Tagawa Y, Yamayoshi S, Kawakami C, Le MQ, Uchida Y, Saito T, Nidom CA, Humaira I, Toohey-Kurth K, Arafa AS, Liu MT, Shu Y, Kawaoka Y. Reactivity and sensitivity of commercially available influenza rapid diagnostic tests in Japan. Sci Rep 2017, 7:14483.	10.1038/s41598-017-14536-0.	published	
Imai M, Watanabe T, Kiso M, Nakajima N, Yamayoshi S, Iwatsuki-Horimoto K, Hatta M, Yamada S, Ito M, Sakai-Tagawa Y, Shirakura M, Takashita E, Fujisaki S, McBride R, Thompson AJ, Takahashi K, Maemura T, Mitake H, Chiba S, Zhong G, Fan S, Oishi K, Yasuhara A, Takada K, Nakao T, Fukuyama S, Yamashita M, Lopes TJS, Neumann G, Odagiri T, Watanabe S, Paulson JC, Hasegawa H, Kawaoka Y. Characterization of a highly pathogenic avian H7N9 influenza virus isolated from an infected human. Cell Host&Microbe 2017, 22:615-626.	10.1016/j.chom.2017.09.008.	published	

6	Total

1. 2 Original Publications (Articles by Single Team only)

All Authors' Names, Title, Journal Name, Volume, Edition, Page, Year of Publication	DOI Code	Publication Status	Remarks (e.g. publication in top level journals etc.)	Country name of the team
Taro Kagaku and Jiro Kagaku、Distinct roles of MLCK and ROCK in the regulation of membrane protrusions and focal adhesion dynamics during cell m	doi: 10.1083/jcb.201506	in press		Thailand
Yamayoshi S, Uraki R, Ito M, Kiso M, Nakatsu S, Yasuhara A, Oishi K, Sasaki T, Ikuta K, Kawaoka Y. A Broadly Reactive Human Anti-hemagglutinin Stem Monoclonal Antibody That Inhibits Influenza A Virus. EBioMedicine 2017, 17:182-191.	10.1016/j.ebiom.2017.03.007.	published		
Yasuhara A, Yamayoshi S, Soni P, Takenaga T, Kawakami C, Takashita E, Sakai-Tagawa Y, Uraki R, Ito M, Iwatsuki-Horimoto K, Sasaki T, Ikuta K, Yamada S, Kawaoka Y. Diversity of antigenic mutants of influenza A(H1N1)pdm09 virus escaped from human monoclonal antibodies. Sci Rep 2017. 7:17735.	10.1038/s41598-017-17986-8.	published		
Yamayoshi S, Ito M, Uraki R, Sasaki T, Ikuta K, Kawaoka Y. Human protective monoclonal antibodies against the HA stem of group 2 Has derived from an H3N2 virus-infected human. J Infect. 2018, 76:177-185.	10.1016/j.jinf.2017.12.004.	published		

3	Total

Lists of Achievements and Implemented Activities

2. presentations at Academic Conferences etc. (Seminars, Workshops, Symposia)

【Notes】
Please fill in **only the achievements of this project** by country in order of presentation date.
The information on this form is only disclosable. Please submit Non-disclosable information in a separate file.

2. 1 Conference Presentations (Joint Presentations among Research Teams)

Date	Type of Presentation	Speaker, "Title", Conference Name, Location, etc.
March 4, 2018	Guest/Invited Speaker	Taro Kagaku, "xxx", yyy, Tokyo,

0 Total

2. 2 Conference Presentations (by Single Team)

Date	Type of Presentation	Speaker, "Title", Conference Name, Location etc.	Country name of the team
March 4, 2018	Guest/Invited Speaker	Taro Kagaku, "xxx", yyy, Tokyo,	Thailand
January 16, 2017	Poster Session	Takeaki Imamura "Analysis of highly pathogenic avian H5N1 influenza viruses isolated in Indonesia" 6th Negative strand virus-Japan, Okinawa, Japan	Japan
March 29, 2017	Oral Presentation	Chairul A. Nidom, "Update of pandemic & H5N1 viruses in Indonesia" Swiss-Indonesia Vaccine Formulation Symposium, Surabaya, Indonesia	Indonesia
March 29, 2017	Oral Presentation	Reviany V. Nidom, "Construction of Indonesian-strain avian flu virus seed vaccine using low pathogenic hemagglutinin gene and neuraminidase PR8 gene through reverse genetic", Swiss-Indonesia Vaccine Formulation Symposium, Surabaya, Indonesia	Indonesia
March 29, 2017	Oral Presentation	Ema Qurnianingsih, "Growth optimization of H5N1 influenza vaccine seed strain Indonesia low pathogenic viruses by reverse genetic in MDCK cell. Swiss-Indonesia Vaccine Formulation Symposium, Surabaya, Indonesia.	Indonesia
March 29, 2017	Oral Presentation	Kadek Rachmawati, "Mutation of H5N1 influenza viruses as a vaccine seed in a variety of hosts by the molecular studies of hemagglutinin and neuraminidase fragment", Swiss-Indonesia Vaccine Formulation Symposium, Surabaya,	Indonesia
January 15, 2018	Poster Session	Takeaki Imamura, Shinya Yamada, Yoshihiro Kawaoka "Analysis of highly pathogenic avian H5N1 influenza viruses isolated in Indonesia"7th Negative strand virus-Japan, Okinawa, Japan	Japan

6 Total

Lists of Achievements and Implemented Activities

3. Workshops, Seminars, Symposia and Other Events （Organized by the Project）

【Notes】
Please fill in **only the achievements of this project** in order of event date.
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Event duration	Name of Organizer	Title of the Event	Location (Country, City, Venue)	Number of Participants (Including Team Members)	Overview
Mar 4-16, 2018	Taro Yamada	○○○○	Germany、Hamburg、○○○○	10	
Mar 7-18, 2016	Shinya Yamada	Training and technical suppport for influenza	Airlangga University, Srabaya, Indonesia	10	gave training for laboratory work
Mar 6-10, 2017	Kiyoko Iwatsuki-Horimoto	Training and technical suppport for influenza	Airlangga University, Srabaya, Indonesia	10	gave training for laboratory work
March 21-30, 2017	Shinya Yamada	Training and technical suppport for influenza	Airlangga University, Srabaya, Indonesia	10	gave training for laboratory work
March 29-31, 2017	Chairul A. Nidom	Swiss-Indonesia Vaccine Formulation Symposium	Airlangga University, Srabaya, Indonesia	145	International Forum for Vaccine
Mar 6-10, 2017	Kiyoko Iwatsuki-Horimoto	Training and technical suppport for influenza	Campus YPN, Surabaya, Indonesia	10	gave training for laboratory work
Mar 22-30, 2017	Shinya Yamada	Training and technical suppport for influenza	Campus YPN, Surabaya, Indonesia	10	gave training for laboratory work
March 8, 2018	Chairul A. Nidom	A New Paradigm for Laboratory Influenza research	Campus YPN, Surabaya, Indonesia	100	Kiyoko Iwatsuki-Horimoto, Shinya Yamada, Takeaki Imamura, and Yukimasa Matsuzawa gave speeches to postgraduate students.

7 Total

Lists of Achievements and Implemented Activities

4. Record of Research Exchanges

【Notes】
Please fill in the record of **resaerch exchange only of this project**.
"Duration of exchange" is not the number of days stayed on the site, but the number of days from departure to return home.
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Date of Departure	Date of Return	Last Name & First Name	Country of Affiliation	Affiliation	Position	Exchange Destination (Country, City, Research Organization etc)	Description of Exchange Content/Purpose	Duration of Exchange (autocompleted)
January 6, 2016	January 16, 2016	Taro Yamada	Japan	Yamada University	Professor	NANOTEC,NECTEC, Bangkok	○○	11
March 7, 2016	March 19, 2016	Shinya Yamada	Japan	Univ. of Tokyo	Assistant Professor	Airlangga Univ., Surabaya, Indonesia	Meeting, training, and assistance for sampling	13
March 7, 2016	March 19, 2016	Imamura Takeaki	Japan	Univ. of Tokyo	Ph.D. student	Airlangga Univ., Surabaya, Indonesia	Meeting and assistance for sampling	13
March 21, 2016	March 31, 2016	Yukimasa Matsuzawa	Japan	Univ. of Tokyo	Ph.D. student	Airlangga Univ., Surabaya, Indonesia	Meeting and assistance for sampling	11
March 4, 2017	March 11, 2017	Kiyoko Iwatsuki-Horimoto	Japan	Univ. of Tokyo	Assistant Professor	Airlangga Univ., Surabaya, Indonesia	Meeting, training, and assistance for sampling	8
March 21, 2017	March 31, 2017	Imamura Takeaki	Japan	Univ. of Tokyo	Ph.D. student	Airlangga Univ., Surabaya, Indonesia	Meeting and assistance for sampling	11
March 21, 2017	March 31, 2017	Shinya Yamada	Japan	Univ. of Tokyo	Assistant Professor	Airlangga Univ., Surabaya, Indonesia	Meeting, training, and assistance for sampling	11
March 1, 2018	March 9, 2018	Kiyoko Iwatsuki-Horimoto	Japan	Univ. of Tokyo	Assistant Professor	Campus YPN, Surabaya, Indonesia	Meeting, lecuture, training, and assistance for sampling	9
March 1, 2018	March 17, 2018	Yukimasa Matsuzawa	Japan	Univ. of Tokyo	Ph.D. student	Campus YPN, Surabaya, Indonesia	Meeting, lecuture, and assistance for sampling	17
March 7, 2018	March 17, 2018	Imamura Takeaki	Japan	Univ. of Tokyo	Ph.D. student	Campus YPN, Surabaya, Indonesia	Meeting, lecuture, and assistance for sampling	11
March 7, 2018	March 17, 2018	Shinya Yamada	Japan	Univ. of Tokyo	Assistant Professor	Campus YPN, Surabaya, Indonesia	Meeting, lecuture, training, and assistance for sampling	11
					Total (Person)	10	Total (Persond-day)	115

Lists of Achievements and Implemented Activities

5. Patent Applications

【Notes】
Please fill in **only the achievements of this project** by country in order of presentation date.
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5. 1 Independent Applications by Single Team

Application Number	Name of Patent/Patent Name	Application Date	Patent Applicants (Fill in All Members)	Publication Number (leave blank if unpublished)	Inventor	Country of Application	Registration Number (leave blank if unregistered)	Country Name of the Team
WO20xx-xxxxxx		January 21, 2016	○○ Univ、Univ.of xx	WO/2016/xxxxxx	○○○○、○○・○○	PCT	WO20xx-xxxxxx (20xx.xx.xx)	Thailand

0 Total (Number of Application)

0 Total (Number of Registration)

5. 2 Joint Applications

Application Number	Name of Patent/Patent Name	Application Date	Patent Applicants (Fill in All Members)	Publication Number (leave blank if unpublished)	Inventor	Country of Application	Registration Number (leave blank if unregistered)
WO20xx-xxxxxx		January 21, 2016	○○ Univ、Univ.of xx	WO/2016/xxxxxx	○○○○、○○・○○	PCT	WO20xx-xxxxxx (20xx.xx.xx)

0 Total (Number of Application)

0 Total (Number of Registration)

Lists of Achievements and Implemented Activities

6. Awards

【Notes】
Please fill in **only the achievements of this project** by country in order of date of Award.
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Date of Award	Name of Award	Recipient	Remarks	Country Name of the Team
December 24, 2015	○○ Prize	Taro Yamada		Thailand

0 Total