e-ASIA Joint Research Program
Progress Report

1. Project Title: Development of new diagnostic tools for detecting malaria parasites resistant to drugs used in Artemisinin-based Combination Therapy

2. Joint Research Period: February, 15, 2019 ～ March, 31, 2022

3. Principal Investigators:

- Japan: Shiroh Iwanaga, Professor, Research Institute for Microbial Diseases, Osaka University
  - Planned Funding Period: February, 15, 2019 – March, 31, 2022
- Thailand: Chairat Uthaipibull, Principal Researcher, Medical Molecular Biotechnology Research Group, National Center for Genetic Engineering and Biotechnology (BIOTEC)
  - Planned Funding Period: in kind
- Indonesia: Din Syafruddin, Professor, Malaria and Vector Resistance Laboratory, Eijkman Institute for Molecular Biology
  - Planned Funding Period: in kind

4. Summary of the Progress of the Joint Research:

Malaria is an important infectious disease that affects populations living in tropical and subtropical regions like Southeast Asian countries including Thailand and Indonesia. At the present, the patients are treated by Artemisinin Combination Therapy (ACT) using artemisinin and partner drugs, such as mefloquine, piperaquine, and lumefantrine. However, the parasites have already acquired resistance to not only artemisinin, but also partner drugs, which make treatment difficult. Therefore, drug resistance of malaria parasite is one of major problem for control of malaria. In this project, we aim to develop the diagnostic method for detecting infection of drug resistant parasites. Especially, we focus on the resistance to antimalarials used in ACT.

(1) Establishment of Drug resistant parasite lines from patients:

We collected blood samples from malaria patients infected with *Plasmodium falciparum* and obtained culturable 200 strains in 2019-2020. *P. falciparum* is the most deadly human malaria parasite. We subsequently examined their drug resistances to the antimalarials used in ACT. Forty drug resistant parasite strains were eventually established: 7 artemisinin resistant strains, 15 mefloquine-resistant strains, 40 chloroquine-resistant strains, and 40 fansidar-resistant strains. One of the established mefloquine resistant parasite strains was used as the sample for identification of novel drug resistance gene by the method using the Plasmodium artificial chromosome (PAC), described in section (3). In addition, established artemisinin resistant parasites are now used as sample for exploration of drug resistance gene by the PAC.

Although the drug resistance to piperaquine and lumefantrine was examined, we could not detect significant resistance among them. This result suggested that the drug resistant parasites to piperaquine and lumefantrine have already emerged in the endemic area, but their extents of spread may be lower than the drug resistant parasites to other drugs, such as mefloquine and chloroquine.

(2) Evaluation of the involvement of known resistance genes in resistance by the CRISPR/Cas9 system:

We investigated the relationship between known drug resistance genes and resistance for the partner drugs. Especially, we focused on mefloquine and piperaquine, because these drugs are widely used in South-East Asian countries including Thailand and Indonesia. In the past epidemiological studies and molecular genetic studies, it was reported that the increase of the copy numbers of PfMDR1, Plasmepsin 2 and 3 confer the mefloquine and piperaquine resistances to the parasites, respectively. Based on these past results, we generated the transgenic parasites in which the copy numbers of those genes were increased by CRISPR/Cas9 system. Briefly, extra copies of PfMDR1 and Plasmepsin 2 and 3 were integrated at the *csp* locus, which was dispensable for development in RBC.

The increase of copy number of PfMDR1 clearly conferred the mefloquine resistance (Fig.1A), supporting the past result. However, the resistance of transgenic parasite with extra copy of PfMDR1 was slightly weaker than well-established mefloquine resistant parasite strain Dd2 which has two copy of PfMDR1, suggesting the presence of other factor involved in mefloquine resistance. The increase of copy numbers of Plasmepsin 2 and 3 did not confer the piperaquine resistance (Fig. 1B). This result suggested that the parasite probably acquires the piperaquine resistance by the factor other than Plasmepsin 2 and 3.

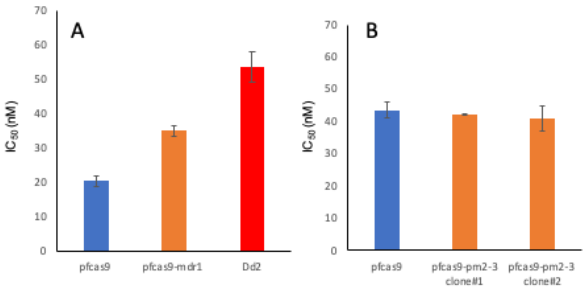

Fig. 1. Mefloquine (A) and piperaquine (B) resistances of transgenic parasites.

(3) Identification of drug resistance gene to the ACT partner drug using an artificial chromosome method:

Mefloquine is the major partner drug used in ACT. In this project, we attempted to identify novel mefloquine resistance gene by functional screening using PAC. To this end, we selected one mefloquine resistant parasite strain, named MEF1, from parasite strains established by us, described in (1). This strain MEF1 exhibits the mefloquine resistance, while it has only single copy of PfMDR1, suggesting that its mefloquine resistance is conferred by gene(s) other than PfMDR1. As an initial step, we generated the genomic library using the PAC from mefloquine-resistant parasites and transfected to the wild-type parasite directly (drug-sensitive). Next, this parasite library was treated with mefloquine at lethal concentration for the wild-type, and the surviving parasites were then harvested. These surviving parasites were considered to acquire mefloquine resistance as a result of introduction of the PAC with the resistance gene. Thus, we recovered PAC from the harvested parasites, and analyzed the incorporated genes. Eventually, a novel ABC transporter, PfMDR7, was identified as the candidate for mefloquine resistance gene. Similar results were obtained in 4 independent experiments, which strongly supported that PfMDR7 conferred mefloquine resistance to the parasites. To further confirm it by genetic engineering approach, we introduced the PAC with PfMDR7 derived from MEF1 into the wild-type parasite. The resultant transgenic parasite clearly acquired mefloquine resistance. Based on this result, we concluded that PfMDR7 is the novel mefloquine resistance gene (in preparation for submission for publication). PfMDR7 is

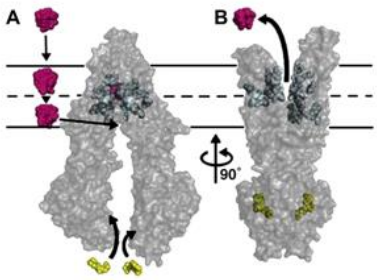

Fig. 2. Model for efflux of lipophilic drug by ABC-B transporter. (Aller, S. G. et.al, Science, 2009)

classified based on the structure into the eukaryotic ABC-B transporter subfamily, which are responsible for the export lipophilic substances. Interestingly, mefloquine is a lipophilic antimalarial drug. Therefore, PfMDR7 may extrude mefloquine from parasite in the conserved manner as ABC-B transporter subfamily, which confers the resistance (Fig. 2).

(4) Development of a diagnostic kit:

(A) The detection system based on LAMP method for infection with drug resistant parasite:

To develop the diagnostic kit for infection with drug resistant parasites, we are attempting to develop the detection system based on LAMP method using chloroquine resistant parasite in pilot experiment. However, due to the difficulty of design the primer set distinguishing the mutations, we were not able to detect the specific mutations of chloroquine resistance gene by LAMP. Now, we re-design the new primer sets to improve the specificity and examine whether they can be used for the detection system. We expect that the system will be developed within FY2021.

(B) The detection system based on q-PCR for infection with drug resistant parasite:

The strain MEF1 harbours only single copy of PfMDR7, and has identical amino acid sequence to the wild-type parasite, but acquires mefloquine resistance by increasing its transcript. Based on these results, we hypothesized that the transcription of PfMDR7 might become a useful indicator for mefloquine resistance. As shown in Fig. 3, increase of PfMDR7 transcription were detected in only mefloquine resistant parasite isolated from patients, but not in sensitive parasites including laboratory reference strain. This result indicates the quantification of transcription of drug resistance gene will become molecular indicator for detecting the infection with drug resistant parasites.

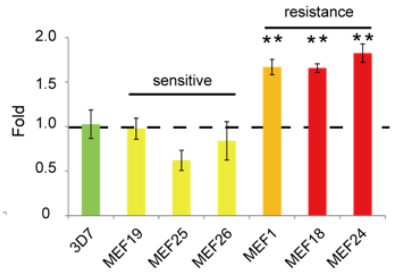

Fig. 3. q-PCR analysis of PfMDR7 in mefloquine resistant (MEF1, 18, 24) and sensitive (MEF19, 25, 26) parasites. The strain 3D7 was used as the reference parasite of mefloquine sensitive parasite.

5. Scientific Achievements and Implemented Activities (Research Exchange, Workshop, Publication, etc. if any):

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

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

Due to the COVID-19 pandemic, it is unclear on the prospect for examination of the developed detection system in field within the project period at the moment. Thus, the short-term goal is to conduct the test of detection system using patient's blood in the field. Furthermore, the long-term goal is the development of diagnostic method based on the result obtained from the test in the endemic area.

7. Recommendations and Comments to the Program (if any):

We think that it will be hard to achieve our goal, described in the section 6, without financial support. Thai and Indonesia groups conduct this project as in-kind, which has been the obstacle. Therefore, we think that the e-Asia members should support financially as much as possible.

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.)

0 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
Aroonsri A, Posayapisit N, Kongsee J, Siripan O, Vitsupakorn D, Utaida S, **Uthaipibull C**, Kamchonwongpaisan S, Shaw PJ. Validation of Plasmodium falciparum deoxyhypusine synthase as an antimalarial target. PeerJ. 2019 Apr 17;7:e6713.	10.7717/peerj.6713	published	publication in top level journals	Thailand
Green JL, Wu Y, Encheva V, Lasonder E, Prommaban A, Kunzelmann S, Christodoulou E, Grainger M, Truongvan N, Bothe S, Sharma V, Song W, Pinzuti I, **Uthaipibull C**, Srichairatanakool S, Birault V, Langsley G, Schindelin H, Stieglitz B, Snijders AP, Holder AA. Ubiquitin activation is essential for schizont maturation in Plasmodium falciparum blood-stage development. PLoS Pathog. 2020 Jun 22;16(6):e1008640.	10.1371/journal.ppat.1008640.	published	publication in top level journals	Thailand
Posayapisit N, Pengon J, Prommana P, Shoram M, Yuthavong Y, **Uthaipibull C**, Kamchonwongpaisan S, Jupatanakul N. Transgenic pyrimethamine-resistant Plasmodium falciparum reveals transmission-blocking potency of P218, a novel antifolate. Int J Parasitol.		in press	publication in top level journals	Thailand
Puji BS Asih, D Syafruddin and J Kevin Baird. Challenges in the Control and Elimination of Plasmodium vivax Malaria", chapter book 4, Towards Malaria Elimination 2018 (Edited by Sylvie Manguin and Vas Dev)	10.5772/intechopen.69750	published		Indonesia

Yamamoto K, Takahashi K, Ato M, Iwanaga S, Ohta N. Antimalarial activity of vitamin D3 (VD3) does not result from VD3-induced antimicrobial agents including nitric oxide or cathelicidin. Exp Parasitol. 2019 Jun;201:67-77.	10.1016/j.exppara.2019.0	published		Japan
Shinzawa N, Nishi T, Hiyoshi F, Motooka D, Yuda M, Iwanaga S. Improvement of CRISPR/Cas9 system by transfecting Cas9-expressing Plasmodium berghei with linear donor template. Commun Biol. 2020 Aug 5;3(1):426.	10.1038/s42003-020-01	published		Japan

6	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.
October 8, 2019	Guest/Invited Speaker	Chairat Uthaipibull, "Identification of resistance markers to antimalarial drugs currently used for malaria treatment", Asia Infectious Disease Project Joint Symposium: Toward the Social Implementation of Health Technology through the Asian Research Network, Jakarta, Indonesia.
August 19-21, 2020	Oral Presentation	Therapeutic Efficacy Studies (TES) of antimalaria DHP-PPQ in Indonesia. Meeting of the Expanded Bangladesh, Bhutan, India, Nepal and Sri Lanka (BBINS) Malaria Drug Resistance Monitoring Network with Indonesia, Maldives and Timor Leste
April 12, 2019	Guest/Invited Speaker	Drug resistance of Malaria parasite: new system for identifying drug resistance gene., Malaria meeting, Leiden, Netherland

3 Total

2. 2 Conference Presentations (by Single Team)

Date	Type of Presentation	Speaker, "Title", Conference Name, Location etc.	Country name of the team

0 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.
The information on this form is only disclosable. Please submit Non-disclosable information in a separate file.

Event duration	Name of Organizer	Title of the Event	Location (Country, City, Venue)	Number of Participants (Including Team Members)	Overview
19-21 August 2020	World Health Organization	Meeting of the Bangladesh, Bhutan, India, Nepal and Sri Lanka (BBINS) Malaria Drug Resistance Monitoring Network with Indonesia, Maldives	Virtual Meeting	54 Participants	

1 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.
The information on this form is only disclosable. Please submit Non-disclosable information in a separate file.

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)
								0
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Total (Person) 0 Total (Persond-day) 0

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.
The information on this form is only disclosable. Please submit Non-disclosable information in a separate file.

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

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)

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.
The information on this form is only disclosable. Please submit Non-disclosable information in a separate file.

Date of Award	Name of Award	Recipient	Remarks	Country Name of the Team
October 1, 2019 – March 25, 2020	Fellowship a for short-term training at the National Center for Genetic Engineering and Biotechnology (BIOTEC)	Farahana Kresno Dewayanti	Research Topic: Identification and validation of drug targets in Plasmodium falciparum	Thailand

1 Total