Molecular analysis of antimalarial drugs resistance in *Plasmodium falciparum* in China

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In China, most malaria drug resistance surveillance relied on *in vitro* microtest and *in vivo* test . In present study, we carried out a study to analysis the molecular markers of antimalarial resistance in *Plasmodium falciparum* in Hainan and Yunnan Province, China.

Since the first report of chloroquine resistance in China was in the early 1970s, chloroquine resistance has been observed in all endemic areas where *in vivo/in vitro* studies have been conducted in the following years. As a consequence of this widespread resistance, a combination of the antifolates sulfadoxine and pyrimethamine was introduced as a second-line treatment of non-complicated malaria in China. The synergistic combination of the two, which inhibits dihydropteroate synthase and dihydrofolate reductase, respectively, in the folate biosynthetic pathway, it was believed that they enhance antimalarial potency and reduce the risk of drug resistance. However, resistance to this drug combination had already been observed in China, and is now wide-spread across the country. The resistance of malaria parasites to Fansidar, the most commonly used combination of sulfadoxine-pyrimethamine, is now widespread in southeast Asia. Following increased CQ and SP resistance, artemisinin and its derivatives gradually became the mainstay of falciparum malaria therapy in China.

Molecular studies over the last few decades have identified several mutations associated with chloroquine and sulfadoxine-pyrimethamine resistance in a number of *Plasmodium falciparum* genes. Polymorphisms in the *Plasmodium falciparum* chloroquine resistance transporter (*pfcrt*) gene, located on chromosome 7, were proposed to be important in chloroquine resistance and transfection experiments have shown that the polymorphism 76-Ser to Thr is tightly linked to the resistance phenotype. Additionally, polymorphisms in the *Plasmodium falciparum* multidrug resistance 1 (*pfmdr*1) gene have been shown by transfection to modulate higher levels of chloroquine resistance and also affect mefloquine, halofantrine, and quinine resistance.

The molecular basis of resistance to pyrimethamine and sulfadoxine has been more clearly defined. Polymorphisms in the dihydrofolate reductase (*dfhr*) gene that alter 108-Ser to Asn/Thr in the enzyme have been shown to confer resistance to pyrimethamine. Additional polymorphisms at amino acid positions 50, 51, 59, and 164 combined with 108-Asn confer increasing levels of pyrimethamine resistance. The combination of 16-Ala to 16-Val and 108-Ser to 108-Thr confers resistance to cycloguanil but retains sensitivity to pyrimethamine. Similarly, polymorphisms in the dihydropteroate synthase (*dhps*) gene confer resistance to sulfadoxine. The polymorphism 437-Gly in *dhps* appears to

be the first to be selected by drug pressure and it encodes lower level resistance to sulfadoxine. Subsequent polymorphisms at positions 436, 540, 581, and 613 confer increasing levels of resistance to this drug. Recently, *Plasmodium falciparum* adenosine triphosphatase-6 (*pfATP6*) S769N mutation has been associated with increased IC50s of artemether.

Epidemiologic studies have been conducted in all malaria endemic areas of the world looking at polymorphisms in the aforementioned genes and their relationship with treatment failure or resistant *Plasmodium falciparum*. The aim of the present study was to complement existing knowledge of *in vivo* and *in vitro* antimalarial drug responses in China by determining the extent of associated gene polymorphisms in *Plasmodium falciparum* isolates from different malaria-endemic areas. The information obtained will contribute to the development of strategies for therapeutic intervention of malaria in China.

METHODS AND RESULTS

Objective

The objective is to analysis of the drug resistance molecular markers based on monitoring efficacy of anti-malarial drugs and to develop a PCR, PFLP and sequencing technique for detection of the durg resistance molecular markers in *Plasmodium falciparum*.

Samples

The *Plasmodium falciparum* samples on the filter paper were collected from China. There were 43 samples with Plasmodium falciparum.

Methods

To design the primers and PCR reaction condition according to relative references. Five drug resistance related genes were amplified by nested-PCR. After amplification, the PCR products of *pfcrt* and *pfmdr* were analyzed by RFLP(restriction fragment length polymorphism), the PCR products of *pfdhfr*, *pfdhps* and *pfATPase6* were sequenced by a biology company.

Results

All of 43 samples with *P.falciparum* were amplified by nested-PCR, *Pfdhfr*, *pfdhps* and *pfATPase6* genes can't be successfully amplified with some samples. The major reason was PCR amplification and getting sequence failure. There were no point mutations found in *Pfmdr1* 1246, *Pfdhfr* 16/108/164, *pfdhps* 613 and *pfATPase6* 769 position. Other

positions have different levels of mutation (Table 1).

Table 1 Summary of drug resistance with five molecular markers

gene	samples	Amino acid position	Sequence base pair size	Sensitive amino acid	Number of sensitive amino acid	Anti amino acid	Number of anti amino acid	Rate of mutation
pfcrt	43	76	145	Lys	5	Thr	38	88%
Pfmdr1	43	1246	203	Asp	43	-	0	0
Pfdhfr	38	16	594bp	Ala	38	-	0	0
		50,51		Cys Asn	14	Cys Ile	24	63%
		59		Cys	3	Arg	35	92%
		108		Asn	38	-	0	0
		164		Val	38	-	0	0
		140		Ile	8	Leu	30	79%
pfdhps	34	436,437	770bp	Ser Ala	0	Ser Gly	3	9%
						Phe Ala	1	3%
						Phe Gly	11	32%
						Ala Gly	9	26%
		540		Lys	3	Glu	31	91%
		581		Ala	16	Gly	18	53%
		613		Ala	34	-	0	0
pfATPase6	32	769	437bp	Ser	32	-	0	0

CONCLUSION

By using the nested PCR, RFLP and sequencing method, we accomplished the analysis of the drug resistance molecular markers in *Plasmodium falciparum* in China. The situation of antimalarial drug resistance in Yunnan Province is more serious than that in Hainan Province. The resistance to chloroquine, sulfadoxine and pyrimethamine still exist in both fields although the use of these drugs has been terminated for a quite long period of time. The molecular marker related to decreased sensitivity of artemisinin can not be found at present in the three fields. The results of the present study improve our understanding of the antimalarial drug resistance situation in China, and provide scientific basis for the establishment and adjustment of the regime of prevention and treatment for falciparum malaria.

2009 Annual report

Construction of international network of research and control on vivax malaria in Asia and Pacific area

SUMMARY AND RECOMMENDATION
2nd INTERNATIONAL CONFERENCE ON VIVAX MALARIA IN ASIA & PACIFIC AREA
SHANGHAI, PR. CHINA, 29-31 JANUARY 2010

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Executive summary

The second international conference on vivax malaria in Asia and Pacific area was organized by National Institute of Parasitic Diseases(NIPD), China CDC and National Institute of Infectious Diseases(NIID), Japan. Forty-five participants were from 16 institutes of 11 countries, (Cambodia, DPR Korea, Indonesia, Japan, Laos, Papua New Guinea, Philippines, Republic of Korea, Thailand, Vietnam and PR China), Roll Back Malaria and two regional institutes of World Health Organization(WPRO and SEARO). The objective of this conference was strengthening inter-country and inter-regional networking for implementation of an evidence-based control of vivax malaria in Asia and Pacific area through exchange of information, experiences and results of operational researches. The participants reviewed the current situation of vivax malaria in Asia and Pacific area, and the recent progress of control programs and researches for vivax malaria in each endemic area, and shared their information and experiences related vivax malarial epidemiology, diagnosis, treatment, prevention and surveillance. The conference focused on the results of operational researches which were prioritized in the first meeting held in 2007. The prioritized researches included an investigation on reliability of clinical diagnosis and rapid diagnostic tests(RDTs) for vivax malaria in the field, a study on development of LAMP to detect vivax malaria parasite in the field and effectiveness of mass chemotherapy with primaquine. The participants confirmed low reliability of clinical diagnosis and RDTs for vivax malaria and necessity of more development of LAMP in the field. The use of primaquine was identified as an effective anti-relapse therapy in the Korean peninsula. This conference recommended close network on vivax malaria in Asia and Pacific area and collaboration with existing other related networks. Based on inter-country collaboration and close networking of institutions, much more promotion of operational researches is necessary for implementation of an evidence-based vivax malaria control program. Priority research areas included: development of RDTs for *P. vivax* and rapid screening test for G-6-PD deficiency and its severity, studies to determine and monitor drug resistance in vivax malaria, and studies to identify prevalence, type, and severity of G6PD in each endemic area. Appropriate use of primaquine is important as a control measurement. Distribution of mosquito vectors should be monitored by a standardized method and effectiveness of LLITN should be evaluated in vivax malaria control program. GIS techniques will be used for studies on the transmission characteristics including climate, vector, parasite and human factors, and development of early warning system.

Background

Malaria is still a public health threat in Asia and Pacific area. *Plasmodium vivax* is the second major *Plasmodiun* spp. causes human malaria, next to *P. falciparum* in the world. *P. falciparum* and *P. vivax* are prevalent in Asia and Pacific area and *P. vivax* is spread from tropical areas in South pacific to temperate areas in the Korean Peninsula. The endemic area of *P. vivax* is much wider than that of *P. falciparum* in this region. Vivax malaria has been generally reported mild and less threat than falciparum malaria. And vivax malaria has been often poorly understood and its burden has been underestimated.

Because of the progress of malaria control programme in the past ten years, mortality and morbidity due to malaria have drastically decreased in the South-east Asia and the South Pacific area. In some areas of this region, main endemic species of *Plasmodium* spp. has shifted from *P. falciparum* to *P. vivax*. This change has resulted in inadequacy of malaria control methods and systems that had been effectively used for many years in the tropical malaria endemic countries. Outbreaks of vivax malaria have been reported in some temperate Asian countries and areas where malaria was once eliminated, in the 1990s and 2000s. Vivax malaria was recognized as one of the common major public health threats in the tropical and temperate Asian countries. An in this situation change, National Institute of Infectious Diseases(NIID), Japan and National Institute of Parasitic Diseases(NIPD), China CDC, P.R. China decided to hold an international conference for construction of inter-country and inter-regional networking on vivax malaria.

Representatives from fifteen institutes of nine countries, (Japan, PR China, Republic Korea, DPR Korea, Cambodia, Thailand, Philippines, Indonesia, and Solomons) and three regional offices of WHO(WPRO, SEARO, and EMRO) participated the 1st international conference on vivax malaria in Asia and Pacific area in Shanghai in 2007. Through the 1st conference, the participants confirmed dynamic changes of malaria situation in Asia and Pacific area and shared many information and experiences on viax malaria. And the multi-country and multi-region network on vivax malaria provided the recommendations including promotion of standardizing surveillance guideline and trainings. For researches, inter-country collaboration was recommended in operational researches related to *P. vivax* epidemiology, diagnosis, treatment and prevention. And as prioritized research topics, the participants selected evaluation of clinical diagnostic

algorithm and RDTS in the field, validation of treatment regimens in each country and primaquine prophylaxis in DPRK and development of LAMP for appropriate use in the field.

After the 1st international conference on vivax malaria in Asia and Pacific area in Shanghai in 2007, malaria control activities have been accelerated in this region. Malaria elimination network in Asia(MalER) launched in Kolombo, Srilanka in 2009. WPRO provided regional action plan for malaria control and elimination in the Westren Pacific(2010-2015) in 2009. There have been a number of meetings and conferences on malaria in this region. To compare the number of conferences on falciparum malaria, the number of those on vivax malaria was previously few. But it is increasing and SEARO held the inter-regional meeting on vivax malaria in Asia in New Delhi, in April 2009, as an informal WHO technical meeting.

In the next steps of malaria control programs towards elimination of malaria, inter-country and inter-region network should be strengthened. Besides, community based specific measurements will be implemented based on the epidemiologic characteristics of each endemic area. Development and evaluation of science evidenced control measurements are necessary in each level of malaria control programs. One of the key issues in control of malaria is frequent relapse that *P. vivax* causes. But, there are knowledge gaps with regard to vivax malaria. And, there are many areas that require both basic and operational researches to control *P. vivax*.

In order to bridge knowledge gaps on vivax malaria, we need continuous feedback from field operations. Through analysis on malarious situation in each endemic country and areas in this network, the participants find obstacles in the control programs and prioritize research topics to solve the problems. The results of researches which are recommended should be carried out and evaluated by using the network. In addition to exchange and sharing of information and experiences in control of vivax malaria, promotion of operational researches to bridge gaps between scientific researches and operations, and implement scientific evidenced control measurements.

Objects of the conference

General objective

To strengthen inter-country and inter-regional networking for implementation of an evidence-based control of vivax malaria in Asia and Pacific area through exchange of information, experiences and results of operational researches.

Specific objectives

(1) To review the current situation of vivax malaria in Asia and Pacific area, and the recent progress of control programme and researches for vivax malaria

(2) To share information and experiences related vivax malarial epidemiology, diagnosis, treatment, prevention and surveillance

(3) To share the results of operational researches which were prioritized in the first meeting held in 2007.

(4) To prioritize research topics and collaborative operational researches through analyzing the current problems for control of vivax malaria

(5) To promote information exchange and strengthen inter-country collaboration and networking of institutions

Prof Tang Linpha(China PR) and Dr Hiroshi Ohmae(Japan) managed the conference as the Chair and Co-Chair, respectively. Dr Jeeraphat was nominated as the rapporteur.

Overview of the first international conference of vivax malaria in Asia of Pacific area and the role of this network to eliminate malaria

Establishment of vivax malaria network in Asia and Pacific area

-Reporting the 1st international conference on vivax malaria in Asia and Pacific area-

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Yayi Guan, Tang Linpha

Malaria remains a major public health problem in the South-east Asia and the Pacific area. The situation of malaria in the past 10 years has improved considerably in this region. But following the successful control programme to reduce the mortality and morbidity due to falciparum malaria, the number of vivax malaria has reversed progressively in some endemic areas. We confirmed that *Plasmodium vivax* has become the dominant species in on-going malaria control endemic projects, in the meeting among National Institutes of the South-east Asian Coutries and Japan, which was held in Tokyo in 2006. Outbreaks of vivax malaria took place in the Korean peninsula in the 1990s and the two countries in the Korean peninsula and the People's Republic of China that also has the same problem have met in Shanghai to exchange the information and progressive experiences in the control.

To compare the thereat of falciparum malaria, that of vivax malaria is silent and it has been neglected for a long time. We recognized vivax malaria as one of common public problems in tropical and temperate Asian countries and National Institute of Infectious Diseases(NIID), Japan and National Institute of Parasitic Diseases(NIPD), China CDC, P.R. China held the 1st international conference on vivax malaria in Asia and Pacific area in Shanghai in January 2007. Representatives of this conference were from fifteen institutes of nine countries; Japan, PR China, Republic Korea, DPR Korea, Cambodia, Thailand, Philippines, Indonesia, and Solomons. Three regional offices of WHO; WPRO, SEARO, EMRO also participated this conference.

In this conference, we shared many results of excellent survey data and confirmed dynamic changes of malaria situation in Asia and Pacific area. And the multi-country, multi-region network on vivax malaria was established to :(1) share information and experience related to *P. vivax* epidemiology, diagnosis, treatment, prevention and

surveillance; and (2) carry out collaborative operational researches on vivax malaria. And the network participants prioritized some research fields such as evaluation of clinical diagnostic algorithm and RDTS in the field, validation of treatment regimens in each country and primaquine prophylaxis and development of new diagnostic methods including LAMP.

Conclusion and Recommendation

Conclusion

- Vivax network is an important network to support controlling until elimination of vivax malaria in the country in Asia Pacific area.
- To Strengthen the network activity by providing evidence, technical information needed, exchanging information and experience to Asia Pacific countries within or outside the network.
- To mobilize resource by using any opportunity available within the Asia pacific countries.

Recommendation:

General

1. To strengthen the network by establishing secretariat in Shanghai PR China in IPD, Chinese CDC.
2. The members in the 2nd International conference on vivax malaria to facilitate and prepare or implement the result of the discussion for operational research in the field of vivax, diagnosis and treatment, surveillance and epidemiology, vector control as attach and presenting in the next 3rd international conference on vivax malaria in Asia pacific in 2012.
3. Secretariat to facilitate the mapping of institutions based on the areas of the respective interest in diagnosis and treatment, surveillance and epidemiology and vector control and identify the lead institution for each area.
4. Secretariat to convene a workshop for working groups on the areas of interest (diagnosis and treatment, surveillance and epidemiology, vector control) in end of this year.
5. To expand the membership by inviting institute, universities, national programme malaria in Asia Pacific and other stakeholders and donors in the 3rd International vivax malaria conference.
6. Secretariat to regularly inform and update the information related vivax malaria network activities and conduct the conference on the year 2012.

Research priorities

The participants were divided to two groups and discussed and agreed on research

priority and collaboration as follows.

Diagnosis

More reliable Rapid Diagnostic Tests, RDTs, for diagnosis of vivax malaria are required. Studies to find out appropriate RDTs for field operation are also needed.

Treatment

2. Studies to determine and monitor *vivax* resistance to chloroquine should be carried out. Molecular marker techniques could be tools of choice to assist identifying of *vivax* resistance. If the *vivax* resistance to chloroquine exists, the studies of appropriate alternative treatment should be carried out by each country.
3. Appropriate total dose of primaquine and shortest duration used on evidence-based manner should be worked out by each country as to achieve effective treatment of vivax malaria in order to minimize liver stage parasites and relapse in each particular location.

Epidemiology and control

1. Studies on Prevalence and Type and Severity of G-6-PD deficiency in each specific country should be conducted (among both *vivax* patients and normal population). Collaborations within countries and across the network regarding technical capabilities are required.

2. Appropriate total dose of primaquine and shortest duration used on evidence-based manner should be worked out by each country as to achieve effective treatment of vivax malaria in order to minimize liver stage parasites and relapse in each particular location. The studies on effectiveness of treatment regarding the spring treatment in China and Mass Drug Administration in DPRK are also required.
3. Studies on development of tools such as adequate molecular markers to differentiate local and imported cases in a community and primary infection and relapse in a person.

4. Evaluation of the effectiveness of LLITN and conventional ITN's, and study on factors influencing for usage of LLIN.

5. Studies on the transmission characteristics including climate, vector, parasite and human factors by using GIS techniques and thereby to improve the stratification of

malarious areas and if possible to develop early warning system based on GIS.

6. Studies on distribution of vector based on an adequate classification of vector species and influencing factors to develop modules for vectors monitoring

In order to develop anti relapse drug and vaccine, we need further basic studies on invasion of Vivax parasite into Liver cells. And to improve the vector control and prevention, we also need further study on survival of parasite in the mosquito vectors regarding ecological impacts.

Ministry of Health
National Centre for Parasitology,
Entomology and Malaria Control
(CNM)

Kingdom of Cambodia
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The Annual Project Report

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The Strengthening & Integrating Of Malaria Control Activities In Newly Developed Area In Kampot Province, Southern Cambodia

Period: September 2009 to February 2010

Date: 28th February, 2010

Submitted by

Dr. Chea Nguon
Dr. Duong Socheat

Supported By

Ministry of Health, Welfare and Labor of Japan (A grant on "Research for emerging and re-emerging infections")

Project Title:

Strengthening and integrating of Malaria Control Activities in newly developed area in Kampot Province, Southern Cambodia.

I. Introduction

Malaria is a major public health problem in Cambodia and a leading cause of mortality and morbidity. It has both short- and long-term consequences for national economic development and has therefore been given high priority by the government and donor agencies. Malaria is the third highest known cause of outpatient attendance (4.6%) and the first cause of hospitalization (13.7%) and hospital death (16%). Real figures are almost certainly much higher as most malaria cases are either treated first through private clinics and drug sellers or do not seek treatment at all.

In Cambodia the malaria transmission happened in the remote forest with little development or nothing and in very poor areas that created complexity in controlling that disease as well as problem of providing and receiving the service delivery from the public health sector. The main problems are that those areas were isolated with the complicated geographical barriers, no roads or roads are very bad or very far away from the health facility that provoked hurdle for the intervention, especially in the rainy season. In addition, the dearth of transportation means, the expensive cost of traveling etc...combined with the limited budget provision for malaria control program made those high endemic and secluded areas separated the public service for many years. Besides the above mentioned, there are still many problems involved and contributed to the low utilization of the public health service.

Responding to this serious problem of malaria, in 2005-2006, with the important grant from Ministry of Health, Welfare and Labor of Japan (A grant on "Research for emerging and re-emerging infections"), the National Malaria Center has piloted the community-based malaria control in six selected newly remote villages in Stung Keo commune , Kampot district, Kampot Province. They are new settlement villages and malaria has been emerging after a few years' non endemic situation.

Through this generous support, the accurate baseline data on malaria incidence and prevalence in those pilot villages were collected. The piloted project have demonstrated that village-based volunteers to provide free of charge service to villagers is the only practical emergency solution for Cambodia to reduce the malaria problem in the remote and inaccessible high endemic communities. With the great success from the first phase in 2005-2007, we still face with another problem in new area in Kampot. So in order to strengthen laboratory network on malaria, we would like to investigate the malaria trends in the newly developed area and compare them among new and previous settlements and other endemic villages, by using new seroepidemiological techniques for genetic analysis on endemic malaria parasites.

II. Project sites

The project sites based in the previous five selected villages (Doung, Malich Kul, Anlong Mac Prang, Trapang Kok, Kampong Chen and Damrei Phong) in Stung Keo commune and the other four new villages(Stung Kbal Domrei, Stung Angkanh, 317, Anlung Krom) in chhouk commune, Kampot district in Kampot province.

III. Purposes of the project

- To get basic epidemiological information based on the comparison of malaria trend in previous and new targeted villages in Kampot province.

- To compare the trend of genes of malaria parasites among previous and new targeted villages in Kampot province, and other malaria endemic villages in Cambodia, by using seroepidemiological methods.

- To strengthen the monitoring of the existing volunteer network with the further integration of other operational and feasible interventions to reduce malaria morbidity and mortality in the newly developed area.

IV. Objectives of the Project

1) Epidemiological comparison in two village groups and other endemic areas

- To oversee the malaria incidence and epidemiological trend in the villages, especially the male adult and children.

- To oversee the dynamics of the malaria parasites in the villages (including seroepidemiological results).

-To compare the genetic dynamics of malaria parasites in two targeted villages and other endemic areas in Cambodia.

2) Activities of the volunteers and village people in the communities

- To integrate and decentralize the re-impregnation activities to volunteers through ITN training with the direct monitoring from the HC, OD and PHD.

- To monitor the volunteers' performance related to the malaria control activities based in the community.

- To strengthen the community's knowledge and practice through the active health education through the community-based network for preventing them from malaria and access them to get the prompt and correct treatment at public health service.