Molecular analysis of chloroquine resistance in *Plasmodium falciparum* in Hainan and Yunnan Province, China

1 Background

The emergence and spread of malaria parasites resistant to many antimalarials, particularly chloroquine (CQ) and pyrimethamine (PY)–sulphadoxine (SD), is largely responsible for the recent resurgence of malaria in China, which severely hampers our capacity to roll back malaria. In China, malaria is still a major public health problem with most of the cases concentrated in two subtropical provinces, Yunnan and Hainan. CQ resistance in *Plasmodium falciparum* appeared in China in the early 1970s and spread rapidly. By the early 1980s, it was replaced by PY–SD to treat falciparum malaria. Since then, longitudinal surveys have been carried out in these two malarious provinces and a general decline of CQ resistance was noticed. In Hainan, *in vitro* microtests demonstrated a drop in the rate of CQ-resistant *Plasmodium falciparum* from 97.9% in 1981 to 60.9% in 1991 and 26.7% in 1997. Correspondingly, 4-week *in vivo* observations detected a decrease of CQ-resistant parasites from 84.2% in 1981 to 40% in 1991 and 18.4% in 1997. Although a similar trend was observed in Yunnan, the decrease of CQ resistance was insignificant and over 90% of *Plasmodium falciparum* remained CQ

resistant in 1992. A more recent survey in 2002 indicated that CQ resistance rate in Yunnan was still above 70%. While this trend of declining CQ resistance as its cessation in many endemic areas suggests a possible rotation of CQ in the future, such a decision requires close regional monitoring because evolution of CQ resistance after the removal of drug pressure seems to differ considerably among parasite populations. Chloroquine resistance is manifested by impaired CQ uptake by the parasite vacuole, which is correlated with mutations in several genes. A single mutation K76T in the *Plasmodium falciparum* chloroquine resistance transporter gene (pfcrt) has been demonstrated to be a major determinant of CQ resistance in *Plasmodium falciparum*. All *Plasmodium falciparum* clinical samples that are resistant to CQ contain the K76T mutation. As at least five founder mutations of K76T have been detected worldwide, genetic backgrounds of the resistant strains differ greatly. Other epistatic markers such as *Plasmodium falciparum* multidrug-resistant 1 (pfmdr1) gene also affect the final outcome of CQ resistance. Five single nucleotide polymorphisms (SNPs) of pfmdr1 N86Y, Y183F, S1034C, N1042D and D1246Y were identified in field isolates from different regions of the world. Some correlation studies showed that 86Y was associated with CQ resistance in isolates from the Old World, whereas the C-terminal mutations were found in isolates from South America. A recent study demonstrated that N1042D of pfmdr1, a

prevalent mutation in South America, contributes to quinine instead of CQ resistance, suggesting that pfmdr1 mutations can affect parasite susceptibility to a wide range of antimalarials depending on the parasite's genetic background. While the effect of pfmdr1 point mutations might affect the parasite's sensitivity to different drugs, recent correlation studies demonstrated that pfmdr1 amplification is responsible for resistance to mefloquine. Currently, the K76T mutation is widely used as a reliable marker for CQ-resistance in epidemiological studies. In different continents, this marker has been shown to correlate with *in vivo* CQ resistance. In China, most malaria drug resistance surveillance relied on an *in vitro* microtest and *in vivo* monitoring. Here, we proposed to carry out a study to analysis the molecular markers of chloroquine resistance in *Plasmodium falciparum* in Hainan and Yunnan Province, China

The National Institute of Parasitic Diseases (IPD), Chinese Center for Disease Control and Prevention (China CDC) is the only specialized institution for parasitology at the national level. Recent years, a series of researches on the molecular markers of antimalarial drug resistance were carried out by the institute, and plenty of experience in the research of the molecular markers was gained. What is more, its senior professional staff, well-established cooperation, extensive researches and great

achievements is the basis of successful implementation of the project.

2 Objectives

(1) To develop a nested PCR-sequencing method for detecting the mutations of *Plasmodium falciparum* chloroquine resistant transport gene (*Pfcrt*)76th codon as well as the mutations of *Plasmodium falciparum* multidrug resistance gene 1 *(Pfmdr1)* 86th codon and 1246 th codon.

(2) To investigate the prevalence of the point mutations in *Pfcrt* K76, *Pfmdr1* N86Y and D1246Y in *Plasmodium falciparum* isolated from Hainan and Yunnan Provinces.

(3) To determine the correlation between the prevalence of mutations in *Pfcrt* K76, *Pfmdr1* N86Y and D1246Y and the level of chloroquine resistance of *Plasmodium falciparum* isolates by *in vitro* microtest.

3 Activities

(1) Blood samples were taken from the cases detected as falciparum malaria in Yunnan and Hainan Provinces.

(2) Nested PCR was developed to amplify the fragments of *Pfcrt* gene including the 76th condon as well as *Pfmdr1* genes including N86Y and D1246Y condon. DNA sequencing and restriction fragment length

polymorphism (RFLP) was used to detect the point mutations.

(3) Chloroquine resistance of the same isolates was measured by the *in vitro* microtest. The prevances of the point mutations in resistant-isolates and that of susceptible ones were compared by x^2 test.

4 Proponent Agency

National Institute of Parasitic Diseases, China CDC, Shanghai , PRC

5 Project Principal Investigator

Prof. Tang Linhua, MD, Director,

National Institute of Parasitic Diseases, China CDC

6 Proposed Budget

Item	RMB	JPY*
Materials and Supplies	45,000	1,194,000
Sample collection	58,000	223,875
Equipment	22,000	298,500
Personnel	11,000	119,400
Communications/ Miscellaneous	7600	164,175
TOTAL	**143,600**	**2,000,000**

(*Conversion rate: 100 JYP = 7.18RMB)

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

Kingdom of Cambodia
Nation Religion King

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

Project Report

Period: September 2008 to February 2009

Date: 28th February, 2009

Dr. Duong Socheat
Dr. Chea Nguon

Supported By

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

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 hyperendemic 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. Through this generous support of General National Institute of Infectious Diseases, Japan for the control activities in the project area, the accurate baseline data on malaria incidence and prevalence in those pilot villages were collected.. The village volunteers were selected and trained for offering the malaria diagnosis and treatment through Paracheck and ACT to the community according to the national guideline. 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 hyperendemic 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 in Asia and the Pacific area, we would like to investigate the malaria trends in the newly developed area by using new epidemiological techniques.

II. Project sites

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

III. Aims of the project

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

- 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

1- To oversee the malaria incidence and epidemiological trend in the villages, especially the male adult and children.
2- To oversee the dynamics of the malaria parasites in the villages

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

1- To integrate and decentralize the re-impregnation activities to volunteers through ITN training with the direct monitoring from the HC, OD and PHD.
2- To monitor the volunteers' performance related to the malaria control activities based in the community.
3- 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.

V. The implementing agency

The National Center for Parasitology, Entomology and Malaria Control will play keys roles in implementing and monitoring the above-mentioned project by coordinating with the full participation from the Provincial Health Department, Operational District, Health Centre, Village Malaria Workers.

VI. Activities and methodology:

A) The formative phase

The preliminary field visits to the new study target areas with the local staffs were conducted in order to discuss the plan of activity and see the real situation in those remote villages. Then the data collection on the existing information of the new targeted villages for the baseline information was carried out. The spleen and household blood surveys were also performed for the new four study villages.

A1- Results of the baseline data collection

- The data collection was conducted in the study villages situated in the Taken commune, Chhouk district, Kampot province. More than 90% of villagers earn their living by farming, hunting, wood and bamboo collection and other forest product. All most of the families in the villages collect the water from the river as their main water source for daily use. Very few latrines in each study village were observed and reported during the survey.

Table 1: Number of population and family in the study villages

No	Name of Village	Total Family	Total Population	Total household	Water Source	Distance from health center
1	Stung Angkanh	416	1596 (416 females)	416	River	40 km
2	Anlung Krom	429	1953 (429 females)	429	River	37 km
3	Stung Kbal Domrei	394	2167 (787 females)	787	River	30 km
4	317	240	643 (240 females)	53	River	38 km
Total		1687	7678	1685		

- A questionnaire related to the malaria signs and symptoms, malaria prevention, treatment seeking behavior was administered to the head of the selected households. The findings could be summarized in the below table:

Table 2: Malaria knowledge of sign/symptom, prevention and treatment seeking behavior

No	Village Name	Know Sign/ Symptom of malaria %	Seek treatment at HC %	Seek treatment at private provider %	Bed net use %	ITN use %	Go to forest
1	Stung Angkanh	67	15	85	52	40	15
2	Anlung Krom	62	12	88	60	50	12
3	Stung Kbal Domrei	59	20	80	66	65	20
4	317	72	25	75	58	61	18

- Spleen survey was conducted in the four study villages in order to splenomegaly and the degree of the endemicity of the local transmission in those villages. In each village 20 children aged from 2-9 years old were screened and assessed for the enlarge spleen. All of them were also tested for malaria by using the rapid diagnosis test (RDT) during the identification. Only three villages were found enlarged spleens and ranked from 10%-20%. The positive RDT test among the three splenomegaly villages also varied from 10%-15%.

Table 3: Results of the spleen survey & blood test among 20 children in each village

No	Village Name	UTM East	UTM North	Forest grade	Ethnicity	Spleen enlarged	RDT+	Spleen grade			
								0	1	2	3
1	Stung Angkanh	48P 0409315	1225246	1	Khmer	3 (15%)	2 (10%)	0	1	2	0
2	Anlung Krom	48P 0409688	1227623	2	Khmer	4 (20%)	2 (10%)	0	3	1	0
3	Stung Kbal Domrei	48P 0409758	1228126	2	Khmer	2 (10%)	3 (15%)	0	1	1	0
4	317	48P 0407519	1230008	3	Khmer	0	0	0	0	0	0

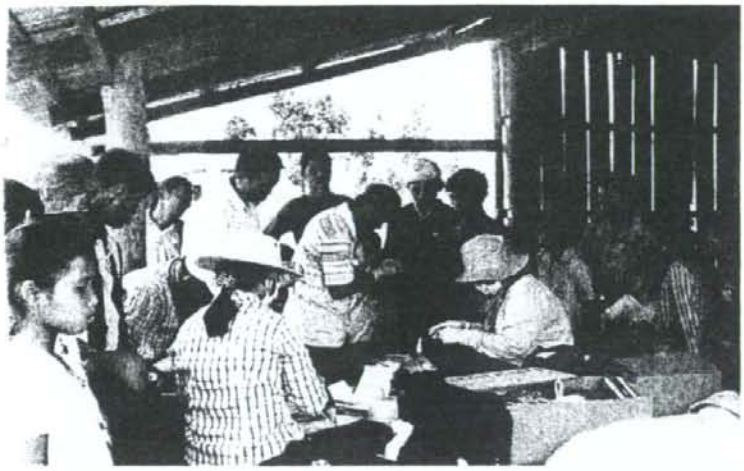
Picture1-4: Spleen Survey activity in the target villages

- The malaria household blood survey also carried out in the four study villages by using the microscopic examination of Giemsa stained blood smears in order to identify the presence of malaria parasites in study villages.

Table 4: Results of the household blood survey

No	Name of Village	No of slide	No of slide positive	P. falciparum	P. Vivax
1	Stung Angkanh	22	4 (18.18%)	2 (9.09%)	2 (9.09%)
2	Anlung Krom	36	10 (27.77%)	8 (22.22%)	2 (5.55%)
3	Stung Kbal Domrei	211	47 (22.27%)	31 (14.69%)	16 (7.58%)
4	317	175	4 (2.28%)	3 (1.71%)	1(0.57%)
Total		**444**	**65 (14.63%)**	**44 (9.90%)**	**21 (4.72%)**

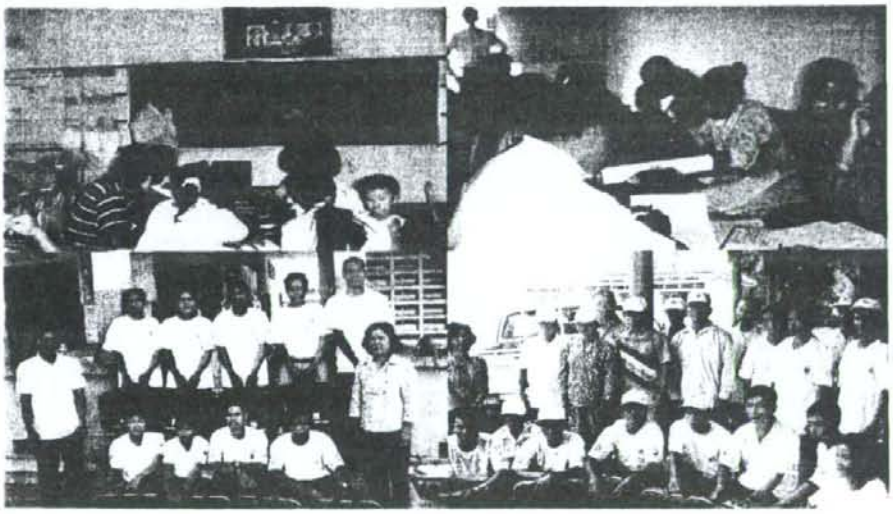
Picture 5: Activity of the household blood survey in the target villages



A2- <u>The selection and training of village malaria data collectors</u>

After collecting the information and conducting the baseline survey in the study villages, 2 villagers per village were selected for implementing the project's activity. The 3day training session organized by the provincial health department collaborated with the national malaria center with the participation from operational district and health center level were provided to the selected village malaria collectors so that they could perform their jobs well for the malaria diagnosis and treatment as well as to collect and monitor the malaria situation in the villages.

Picture 6-9: Training activities and the village malaria data collector trained



B- Monitoring of the malaria cases in the study villages

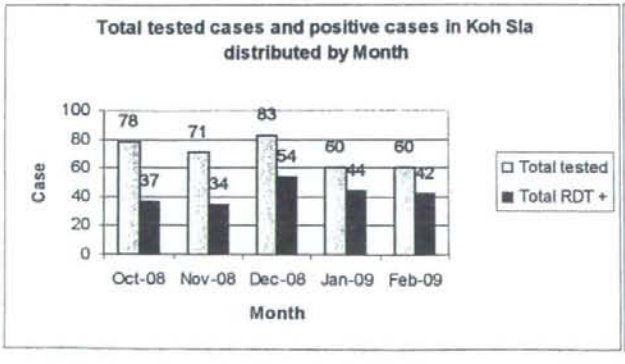
The monitoring and supervisory visits have been made regularly on the monthly basis by the provincial coordinator and from central level to ensure the quality of the data collection as well as to offer the technical support to the village malaria data collectors. The supply also provided to them including anti-malaria drugs and rapid diagnosis tests for the implementation of the activities. During the project implementation, 635 cases were tested and 395 cases were positive and treated in the target villages. The positive rate was 62.20%. In Koh Sla and in Stung Keo the positive rate were 60% and 65% respectively. The data collected from the villages could be depicted in the following tables:

Table 5: Data collected from the project villages in Koh Sla & Stung Keo

Village	RDT Results		Age 0-4		5-14 Y	Age 15-49				Age >=50		
	Total tested	Total RDT +	RDT VE +	RDT VE -	RDTV E +	RDT VE -	M+	F+	RDT VE -	M+	F+	RDT VE -
Kbaldomrey	128	70	4	0	26	9	26	7	45	1	1	4
Angkanh	81	52	2	1	6	4	27	12	24	4	1	0
Anglungkrom	97	60	2	0	18	7	21	17	29	2	0	1
317	46	29	0	0	3	3	16	7	13	2	1	1
Total	**352**	**211**	**8**	**1**	**53**	**23**	**90**	**43**	**111**	**9**	**3**	**6**

Village	RDT Results		Age 0-4		6-14 Y	Age 15-49				Age >=50		
	RDT tested	RDT +	RDT +	RDT -	RDT+	RDT-	M+	F+	RDT-	M+	F+	RDT-
Anglungmakprang	72	55	0	0	4	1	49	0	16	2	0	0
Dong	16	4	0	0	0	1	2	1	11	1	0	0
Kampongchen	42	27	0	0	2	0	23	1	13	1	0	2
Malickul	53	38	1	0	1	0	33	0	14	3	0	1
Domreyphong	54	27	0	0	2	0	19	3	26	3	0	1
Trapangkok	46	33	0	0	1	3	26	5	10	0	1	0
	283	**184**	**1**	**0**	**10**	**5**	**152**	**10**	**90**	**10**	**1**	**4**

Figure 1-2 : Monthly monitoring malaria data collected in the project villages



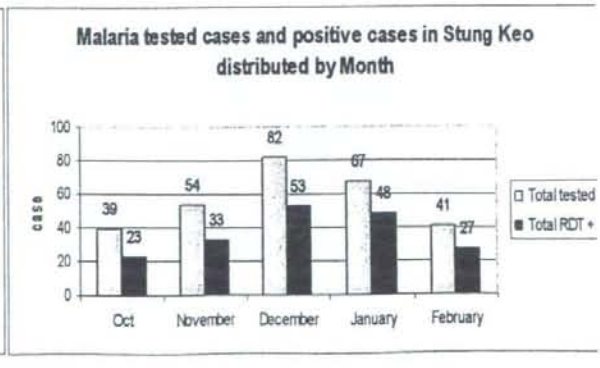
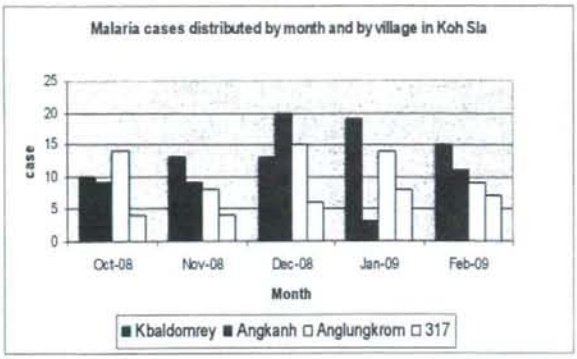


Figure 3-4: Monthly malaria cases distributed by month and village in Koh Sla and Stung Keo



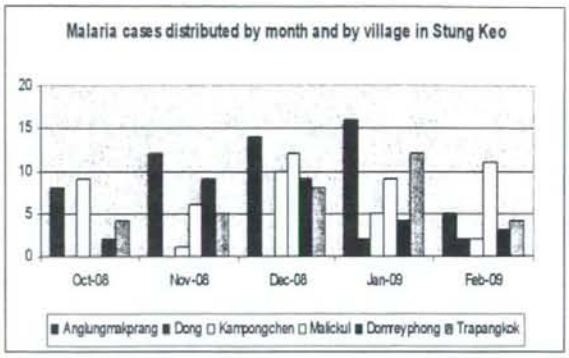
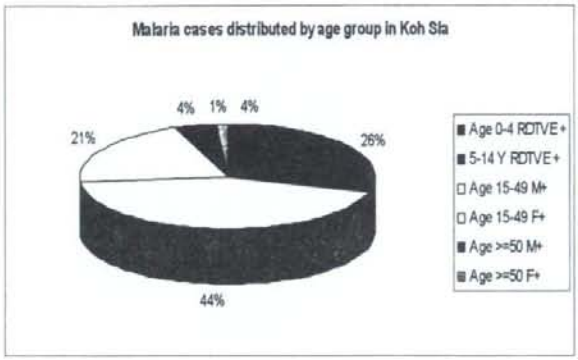
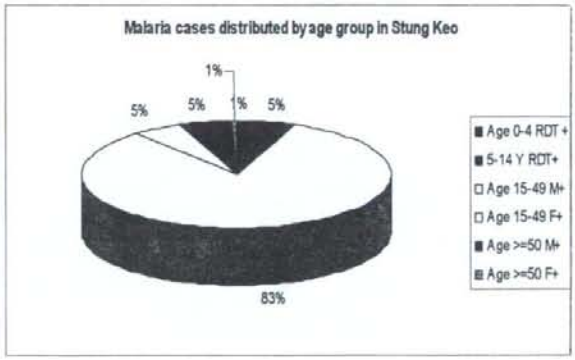


Figure 5-6: Malaria cases distributed by village in Koh Sla and Stung Keo





C- <u>The monitoring of the net distribution and reimpregnation</u>

Personal prevention and protection against malaria's transmission through the insecticide treated nets have been considered as the main priority and effective measure for the malaria control's strategy. During the period of the project implementation 6,661 new bed nets were distributed to the study villages with 2,179 old nets, were retreated by health centers collaborated with village worker and monitored by the project officers to ensure the correct utilization and the appropriate net keeping after use. The percentage of net coverage for risk population in each village is more than 100% with 2.3 people per net's protection criteria. The net received per family ranged from 1.44 to 5.90 per family and the average number of nets received per family is around 2.6 nets per family. The community actively involved in the bed net re-impregnation sessions conducted by the volunteers since they start to aware the importance of the insecticide treated net for preventing themselves as well as to protect their families.

Table 6: Number of bed nets distributed and re-impregnated

Village	No.of family	Pop. at risk	Net distribution	Net Retreatment	Total	% of coverage	Nets received per family
Stung Kbal Damrei	394	2167	1000	365	1365	126	2.54
Stung Angkanh	416	1596	600	200	800	100	1.44
317	240	643	600	266	866	269	2.50
Ang krom	429	1953	850	275	1025	105	1.98
Kampong Chen	236	1300	751	140	891	137	3.18
Trapeang Kak	159	876	341	269	610	139	2.14
Doung	253	1391	721	204	943	136	2.85
Malich Kol	110	605	298	104	402	133	2.71
Anlong Meakprang	253	1372	1075	233	1308	191	4.25
Damrei Phong	72	397	425	123	548	276	5.90
Total	**2562**	**12300**	**6661**	**2179**	**8758**	**142**	**2.60**

Picture10-11 :Activities of the bed nets distribution in the community

D- Strengthen community's knowledge & practice through active health education

Raising awareness through health education for malaria prevention is one of the important components in the implementation villages to prevent the villagers from mosquito bite and transmission to other people. During the project period, there is the total of 150 health education sessions were provided to the study villages and more than 12,000 villagers educated through the sessions. Leaflets, flipcharts and posters were also distributed via village malaria collectors as part of the health education strategy.

Picture 12-13 : Activities of the health education session provided to the community

VII- Findings and discussion

1. Among 4 new selected villages in Koh Sla for the project implementation, 3 of them were found 9 enlarged spleen children age from 2-9 years old and also had 7 children were positive confirmed by RDTsduring the spleen survey with spleen rate and positive dipstick from 10-20% and 10-15% respectively. So the local transmission could be existed in the newly selected villages due to the result received.

2. Many villagers go to the forest for collecting the forest product due to the economic problem and most of them stay at night so that the chances of getting malaria are very high according to the low knowledge on malaria education prevention and the delay of the health treatment seeking behavior. The adult male villagers, particularly, are the most exposed people to malaria in these areas because they are the breadwinners of the family.

3. According to the findings, less than 26% of the villagers seek treatment at the public health facility due to the geographical barrier together with less or little awareness of the disease.

4. The monthly malaria trend of the old and new villages vary according to the malaria transmission season but in overall both areas have the similar trend due to the result of the data collected.

5. The monthly average malaria positive rate in the study villages during the period of the project implementation ranges from 23%-76% in Stung Keo and 55%-65% in Koh Sla.

6. The villages in Stung Keo have higher positive rate (65%) than villages in Koh Sla(60%) compare to the total positive cases. The most affected group is the male aged from 15-49 years old in both Stung Keo (83%) and Koh Sla (44%). However affected agegroup 0-4 years old, 5-14 years old and 15-49 years old female in Koh Sla have more malaria cases than Stung Keo with 4%, 26%, 21% and 1%, 5%, 5% respectively.

7. There is no much significant different percentage of malaria cases between Koh Sla (91%) and Stung Keo (93%) for all patients in the agegroup from 5-49 years old if

regardless of the gender consideration.

VIII- Conclusion and recommendation

- The Strengthening and integrating of malaria control activities in newly developed areas in Kampot Province, Southern Cambodia is an important intervention and greatly contributed to the malaria control in the country.
- The monitoring of the epidemiological information in those endemic areas has provided the interesting information for the prompt intervention and follow-up of the malaria's transmission trend is very useful for the evaluation of the post intervention.
- .The comparison of malaria tendency between the previous and new targeted villages will be clearly explained about the malaria epidemiological change of the affected age group living in the different settings that need for the different kind of intervention in order to address to those typical situation.
- Since the malaria burden in the project areas is still very high, so the continuation of the project monitoring and intervention are very essential so as to see the future shift of malaria epidemiology for the next few years.

IX. Acknowledgements

- Taking this great opportunity, the National Center for Parasitology, Entomology and Malaria Control would like to thank to the Ministry of Health, Welfare and Labor, Japan, for the kind and continuous assistance to our Center.
- A heartfelt gratefulness also to National Institute of Infectious Disease, Japan for their technical support and facilitation for the grant application.
- A profuse appreciation as well to all the collaborators and staff at all levels who have contributed their time and endeavor to make the project functioning fruitfully.

Annual Report for October 2008- Match 2009

Proposal title: Screening of transmission blocking efficacy of antibodies produced against *Plasmodium vivax* and *P. falciparum* vaccine candidates.

Objectives:

1. Identify *Plasmodium falciparum* and *P. vivax* transmission-blocking vaccine candidates.

2. Screening for transmission blocking efficacy of animal antibodies produced against the vaccine candidates by membrane feeding assay

Introduction:

Transmission-blocking vaccines (TBVs) prevent the transmission of malaria by inducing antibodies against antigens specifically expressed on the sexual stage parasites. Since well-characterized TBV candidates are only four (Pfs25, Pfs28, Pfs48/45, Pfs230), it would be necessary to prepare as many novel TBV candidates as possible for making the TBV development successful. In order to identify the novel TBV candidates, Dr. Tsuboi and his group has established a post-genome approach using wheat germ cell-free protein expression system. Based on searching the combined datasets between genome and transcriptome databases, genes that are expected to be expressed in gametocyte stage of *P. falciparum*, have been selected, templates for transcription through PCR-based procedures have been prepared and followed by high throughput recombinant protein synthesis by wheat germ cell-free system. For *P. vivax* the *P. falciparum* orthologs genes will be selected. Many proteins will be produced and used to immunized mice and rabbits. Antibodies raised against these proteins will be screened for blocking of *P. falciparum* or *P. vivax* development in the mosquitoes.

Progress for this reporting period:

1. Standard operating procedures (SOP) for blood collection and preparation for standard membrane feeding assay (SMFA) have been established. The SMFA will be performed once serum from animal (mice or rabbit) immunized with vaccine candidates will be obtained and send to AFRIMS in the following years.

2. *Plasmodium falciparum* gametocyte antigens for immunofluorescent screening of the vaccine candidates were prepared from the *in vitro* culture of the parasites.

3. *Plasmodium vivax* gametocyte and ookinete antigens were prepared from patients blood and infected mosquitoes to be used for western blot and immunofluorescent screening of *P. vivax* vaccine candidates.

Plan for 2009:

1. Validation of SMFA will be performed.
2. After SMFA is validated, the SOP will be followed to screening of the transmission blocking efficacy of the serum samples collected from animal immunized with the vaccine candidates.
3. *Plasmodium falciparum* and *P. vivax* antigens for immunofluorescent assay and western blotting will be prepared from blood stage (gametocytes) and mosquito stages (ookinetes and sporozoites) parasites.

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Genetic Evidence for *Plasmodium falciparum* Resistance to Chloroquine and Pyrimethamine in Indochina and the Western Pacific between 1984 and 1998

Yumiko Saito-Nakano,* Kazuyuki Tanabe, Kiseko Kamei, Moritoshi Iwagami, Kanako Komaki-Yasuda, Shin-ichiro Kawazu, Shigeyuki Kano, Hiroshi Ohmae, and Takuro Endo

Department of Parasitology, National Institute of Infectious Diseases, Tokyo, Japan; Laboratory of Malariology, International Research Center of Infectious Diseases, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan; Faculty of Human Care, Teikyo College, Tokyo, Japan; Department of Appropriate Technology Development and Transfer, Research Institute, International Medical Center of Japan, Tokyo, Japan; Research Unit for Advanced Preventive Medicine, National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Hokkaido, Japan

Abstract. *Plasmodium falciparum* resistance to chloroquine and pyrimethamine is widely distributed in malaria-endemic areas. The origin and geographic spread of this drug resistance have been inferred mainly from records of clinical resistance (treatment failure). Identification of the *Plasmodium falciparum* chloroquine resistance transporter (*pfcrt*) gene and the dihydrofolate reductase (*dhfr*) gene as target genes of chloroquine and pyrimethamine, respectively, has made it possible to trace the history of genetic resistance to these two drugs. However, evidence for genetic resistance has been limited because of scarcity of archival specimens. We examined genotypes of *pfcrt* and *dhfr* in Indochina (Thailand, Myanmar, and Laos) and the Western Pacific (the Philippines, Indonesia, and Papua New Guinea) between 1984 and 1998 by testing samples obtained from malaria cases imported to Japan. Results show that 96% (28 of 29) and 77% (20 of 26) of samples had resistant genotypes of *pfcrt* and *dhfr*, respectively, substantiating the inferred history of clinical resistance in these geographic areas during this period.

INTRODUCTION

Drug resistance imposes a serious burden in the treatment of *Plasmodium falciparum* malaria in tropical regions. Chloroquine (CQ) resistance in *P. falciparum* first appeared almost simultaneously in Southeast Asia and South America in the late 1950s.[1] In Southeast Asia, failure of CQ treatment was reported in all countries in Indochina during the 1960s, with CQ resistance rapidly reaching the Western Pacific and Africa by the early 1970s. CQ resistance is currently prevalent in all malaria-endemic areas, except the Caribbean, China, and the Middle East.[2]

Sulfadoxine-pyrimethamine (SP) was used as an alternative drug for treatment of CQ-resistant malaria in Indochina in the late 1960s.[3] However, resistance to SP rapidly emerged in malaria-endemic areas where CQ was replaced with SP.[4] In Thailand, SP was used as a first-line treatment in the early 1970s,[5,6] but it was abandoned in the early 1980s because of increased treatment failures.[6,7] Laos and Myanmar also introduced SP in the early 1970s, but stopped its usage recently.[6] SP was rarely used in Cambodia, Vietnam, and Malaysia for treatment of CQ-resistant malaria, but resistance to SP has also been prevalent in these countries.[4,6] Resistance to SP was also observed in the Western Pacific countries of Indonesia, Papua New Guinea, and the Philippines in the 1980s.[4] Thus, SP resistance is now highly prevalent in Indochina and the Western Pacific.[2]

The molecular basis of CQ and pyrimethamine (Pyr) resistance has been well characterized in *P. falciparum*.[8] The target gene of CQ resistance is the *P. falciparum* CQ resistance transporter (*pfcrt*) gene, which encodes a putative transporter localized in the digestive vacuole membrane of the parasite. Among 10 amino acid substitutions within *pfcrt* identified among drug-resistant parasites, replacement of lysine with threonine at amino acid 76 is essential for conferring resistance to CQ.[8] The target gene of Pyr is the dihydrofolate reductase (*dhfr*) gene, which encodes dihydrofolate reductase, a key enzyme in the folate biosynthetic pathway. Six amino acid substitutions within *dhfr* have been reported among Pyr-resistant parasites.[9,10] An essential mutation for resistance is a substitution of serine with asparagine at amino acid 108.[10,11] Additional point mutations (at 16, 51, 59, and 164) are associated with increased levels of Pyr resistance *in vitro*.[10,11]

Sequence polymorphisms occur in the *pfcrt* and *dhfr* loci in field isolates of *P. falciparum*, and distributions of polymorphisms differ among geographic areas.[12–15] Confining the polymorphisms to Indochina and the Western Pacific, a *pfcrt* genotype of CVIET at positions 74, 75, and 76 (mutated residues are underlined) represents the most common CQ-resistant type in Indochina, and a *pfcrt* genotype carrying SVMNT at positions 72 and 76 is observed predominantly in the Western Pacific.[12,13] In the Pyr-resistant *dhfr* polymorphism, triple (CIRNI at 51, 59, and 108) and quadruple (CIRNL at positions 51, 59, 108, and 164) mutants are prevalent in Indochina, and the double mutant (CNRNI) is the major *dhfr* genotype in the Western Pacific.[14,15] Migration of genotypes conferring resistance to CQ and Pyr from Indochina to Africa have also been demonstrated.[16,17]

Previous evidence for drug resistance of *P. falciparum* has been derived from records of clinical resistance (treatment failure) and/or *in vitro* drug sensitivity tests. Obtaining genetic evidence for resistance to CQ and Pyr was not feasible until after the identification of target genes and mutation(s) involved in the drug resistance.[4] It should be emphasized that genetic resistance and clinical resistance are not always consistent because selection of drug-resistant parasites results from the interplay of the parasite, drug, and human host, and is largely influenced by immune factors and the pharmacokinetics of the drugs.[18,19]

In areas highly endemic for malaria, such as tropical Africa, CQ and Pyr are still effective, although only partially, in persons infected with drug-resistant genotypes largely because of their immunity, which is acquired after repeated infec-

* Address correspondence to Yumiko Saito-Nakano, Department of Parasitology, National Institute of Infectious Diseases, Fault, Toyama 1-23-1 True, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan. E-mail: yumiko@nih.go.jp

tions.[20,21] In addition, discrepancies between *in vitro* test results and genotypes have been reported: isolates carrying resistant genotypes sometimes show susceptibility to drugs, and *vice versa*.[22] Thus, the history of *P. falciparum* genetic resistance to drugs remains elusive. We therefore consider it is important to obtain genetic evidence of *P. falciparum* drug resistance to further understand the history of parasite resistance to these drugs.

In this study, we investigate polymorphisms in the *pfcrt* and *dhfr* loci in Indochina and the Western Pacific between 1984 and 1998 using archival samples. Results obtained show a high prevalence of resistance to CQ and Pyr during this period, extending back before genetic resistance was first reported in these areas, and substantiating the inferred history of clinical resistance in these geographic areas during this period.

MATERIALS AND METHODS

Parasite samples. Blood samples used in this study were collected as part of a national surveillance system of imported malaria cases in 50 hospitals in Japan between 1984 and 1998, and stored as blood smears. All cases were diagnosed by examination of Giemsa-stained blood smears by two experienced microscopists. The total number of samples examined was 588, with 30–60 samples per year. Annual reported cases of imported malaria in Japan were approximately 100 during this period.[23] Among the 588 cases, 229 samples were positive for *P. falciparum*, 347 for *P. vivax*, 7 for *P. ovale*, and 5 for *P. malariae*. Of the 229 *P. falciparum* samples, we examined 55 single *P. falciparum* infections that originated in Southeast Asia and the Western Pacific in this study. The remaining 174 cases from Africa, South Asia, and South America will be analyzed elsewhere.

Extraction of DNA. Parasite DNA was extracted according to the method of Kimura and others.[24] Briefly, Giemsa-stained slides were dipped in xylene and then in methanol to remove the immersion oil and dye. Each blood smear was scraped off a destained slide with an edge of a clean glass slide, and subjected to DNA purification using QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). To avoid cross-contamination of parasite DNA sample by sample, each scraping off was done using a fresh glass slide on a plastic dish, which were disposed immediately after scraping off. A non-infected blood smear was also used as a negative control in the DNA extraction and polymerase chain reaction (PCR) amplification procedures. The purified DNA, eluted in 100 μL of elution buffer provided with the kit, was stored at 4°C.

Polymerase chain reaction. We amplified a 190-basepair region in the second exon of *pfcrt*, which contained the polymorphic regions of amino acid residues 72–76, by using the nested PCR method described by Djimde and others.[25] Primers were the same as those previously described.[25] For amplification of *dhfr*, we targeted three regions of approximately 190 basepairs that covered four polymorphic residues involved in Pyr resistance. The PCR amplification was performed using Phusion™ high-fidelity DNA polymerase (New England Biolabs, Beverly, MA) in a 50-μL reaction mixture containing 1 μL of extracted DNA, 1 μL (10 mM) of each dNTP mixture (0.2 mM each), 10 μL of 5× Phusion HF buffer, 0.5 μL of DNA polymerase, and 0.5 μM of primers described below.

The regions and primers used were 1) a fragment flanking amino acids 51 and 59 with outer primers 21F (5′-GCC ATA TGT GCA TGT TGT AAG GTT GAA AGC-3′) and 22R (5′-CTT ATA TTT CAA TTT TTC ATA TTT TGA TTC-3′), and inner primers 23F (5′-TGT TGT AAG GTT GAA AGC AAA AAT GAG GGG-3′) and 24R (5′-TTT TTC ATA TTT TGA TTC ATT CAC ATA TGT-3′); 2) a fragment flanking amino acid 108 with outer primers 25F (5′-TGT AAA TAT TTA AAC AAA GAA ACT GTG GAT-3′) and 26R (5′-TTC ATC AAA ATC TTC TTT TTT TAA GGT TCT-3′), and inner primers 27F (5′-GAA ACT GTG GAT AAT GTA AAT GAT ATG CCT-3′) and 28R (5′-TTC TTT TTT TAA GGT TCT AGA CAA TAT AAC-3′); and 3) a fragment flanking amino acid 164 with outer primers 29F (5′-GAA GAT TTT GAT GAA GAT GTT TAT ATC ATT-3′) and 33R (5′-AAA TAC ATC ACA TTC ATA TGT ACT ATT TAT-3′), and inner primers 31F (5′-GTT TAT ATC ATT AAC AAA GTT GAA GAT CTA-3′) and 34R (5′-ACA TTC ATA TGT ACT ATT TAT TCT AGT AAA-3′). The PCR conditions to amplify the *pfcrt* and *dhfr* fragments were 98°C for 30 seconds, then 98°C for 10 seconds, 45°C for 20 seconds, and 72°C for 20 seconds for 40 cycles for the first amplification, and 98°C for 30 seconds, then 98°C for 10 seconds, 50°C for 20 seconds, and 72°C for 20 seconds for 30 cycles for the second amplification. Amplified fragments were subjected to direct sequencing with an ABI3730xl DNA sequencer (Bio Matrix Research, Inc., Tokyo, Japan). One sample isolated from the Philippines in 1998 had a mixed infection, showing both a wild-type and CQ-resistant *pfcrt* genotype (CVIET), as detected by double peaks in an electropherogram. Sequences were confirmed by sequencing two independent amplicons obtained from individual original templates.

RESULTS

Polymorphism in *pfcrt*. Of the 55 samples subjected to PCR, amplification of the *pfcrt* fragment was successful for 29 samples. The low rate of successful amplification was probably caused by low parasite numbers in the blood smears, many of which had parasitemias < 0.03%. Thus, the success rate was 64% for samples with parasitemias > 0.03%, and 33% for those samples with parasitemias < 0.03%, which is consistent with a low rate of successful amplification using blood smear samples with low parasitemias.[24] The polymorphisms observed are shown in Table 1. Records of previously reported *pfcrt* genotypes are combined with the present results and shown in a time-line scheme (Figure 1A). All of our samples showed a CQ-resistant *pfcrt* genotype, with the exception of a sample isolated from the Philippines in 1985. The CVIET CQ-resistant *pfcrt* genotype was present in samples from Myanmar and Laos collected in 1994. This date is five years earlier than the first reported record of this CQ-resistant *pfcrt* polymorphism in those two countries.[6]

In samples from Thailand, the CVIET genotype was detected in 1984, 1991, and 1992. An earlier presence of this resistant genotype has been noted in some culture-adapted parasite strains isolated from Thailand: the K1 strain isolated in 1979,[26] the Indochina III strain in 1984,[27] and the TM284 strain in 1990.[28] The prevalence of this CQ-resistant genotype was 100% in 1995 in Thailand.[6,29] Thus, our results suggest