ກະຊວງ ສາທາລະນະສຸກ
Ministry of Health

ສະຖາບັນ ປັດສະເຕີ ລາວ
INSTITUT PASTEUR DU LAOS

Institut Pasteur du Laos

Activities Report 2015-2016

Réseau International des Instituts Pasteur

Summary

Mandate

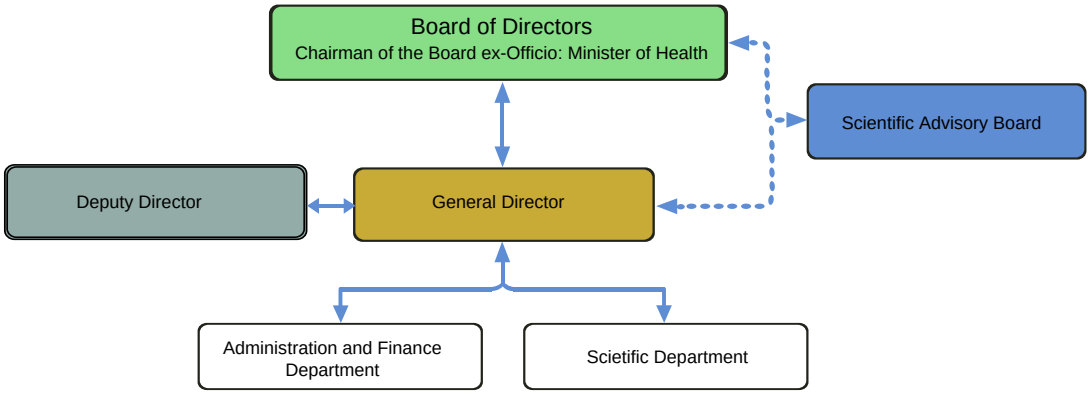
Institut Pasteur du Laos (IPL) is a Lao National Institution created by Prime Ministerial Decree in November 2007 IPL is the result of a long term and joint decision between Lao Ministry of Health and Institut Pasteur Paris which commits to stay 16 years before retroceding the full management of IPL. Sustainability will be achieved by preparing a new generation of Lao doctors and scientists to fill key positions as heads of laboratories and administration at IPL.

IPL has a mandate from Lao Ministry of Health to fulfil activities of public service :
1. Research and diagnostics on emerging infectious diseases and vector borne diseases.
2. Training, education and capacity building.
3. Technical assistance to National Center for Laboratory and Epidemiology (NCLE) for investigation of epidemics.

IPL benefits from a large degree of autonomy (legal, scientific, management, financial) and as such can be considered as a new model of Lao public institution. All the ownership belongs and remains the property of Lao PDR.

IPL has a scientific autonomy within its mandate provided by the MoH. It is able to engage freely in collaborative research and investigations with other Lao and international research and public health organisations.
Financial issues are independent from the Lao public finance system. IPL is able to receive outside funding (donations, grants, bequeaths, etc.) and to generate its own resources through its own discoveries to insure its sustainability.

Main Organigram



IPL is governed by a Board of Directors composed of 3 Lao Members appointed by the Lao Ministry of Health and 2 members appointed by IP Paris. A specificity of the Board meetings is the participation of the main contributors and stakeholders as observers in the spirit of transparency and partnership.

Actual composition of the Board of Directors:

Pr. Dr. Eksavang VONGVICHIT, (Chairman), Minister of Health, Lao PDR.
Dr. Ponmek DALALOY (Honorary Chairman of the Board), Former Minister of Health, Lao PDR.
2 new members to be named by the Ministry of Health of Lao PDR Pr. Dr. Didier SICARD, Honorary President of the National Ethic Committee of France, France. Dr. Marc JOUAN, Secretary-General of the Institut Pasteur International Network, France.

Actual composition of Scientific Advisory Board:

Prof. Felix Rey, Institut Pasteur, Paris.
Prof. Olivier LORTHOLARY, Necker Hospital, Paris.
Dr. Samlane POMPIDA, Former Director National Center for Malariology, Parasitology and Medical Entomology, Ministry of Health, Lao PDR.

Letter from

Dr. Paul BREY

Director

During the past year, IP Laos has considerably consolidated its laboratory staff as well as its laboratory and training infrastructure. As of September 2014 IP Laos has a total staff of 53 persons of whom 43 are Laotian. In line our original plan, established in 2007, IP Laos is now fully staffed and functional with all laboratories occupied. In addition, an exceptional donation on the part of the Henry M. Jackson Foundation with support from US Department of Defense Global Emerging Infections System (GEIS), allowed IP Laos to fully equip its dedicated BSL1 & BSL2 training laboratories. The training laboratories provide a unique facility in Lao PDR and will contribute to the training of Lao medical doctors and Ministry of Health staff from the capital and the provinces.

In line with its National Institute status, IP Laos continues to work hand in hand with the Lao Ministry of Health providing evidence-based data to the Lao MOH on a weekly basis. To facilitate this communication Dr. Darouny Phonekeo was officially appointed as Deputy Director of IP Laos by the Minister of Health. Dr. Darouny's presence facilitates the communication and information transfer between IP Laos and the Ministry. Following the serious dengue epidemic of 2013, the Government of Lao PDR and other stakeholders such as the World Health Organization have recognized the Arbovirus and Emerging Viruses Lab of IP Laos for their excellence and tireless efforts in the area of dengue, surveillance, diagnostics and serotyping.

During the year, IP Laos also welcomed Dr. Shigeyuki Kano and his team from the National Center for Global Health and Medicine (NCGM) Tokyo, who set up the Lao Japan Lab focusing on Malaria drug resistance and trematode infestations with the support of the Japan International Cooperation Agency (JICA) and Japan Science and Technology Agency. The Lao-Lux lab continues to contribute important finding on vaccine preventable diseases and has carried out several projects with the Lao Blood Bank, Ministry of Health and Luxemburg Development to improve disease surveillance, better monitor vaccination coverage and efficacy. The IP Laos Medical Entomology Lab continues its ongoing programs with the US Naval Medical Research Center Asia (NMRC-A), the French Ministry of Foreign Affaires and French Development Agency (AFD – ECOMORE) to identify medically important arthropod vectors, in specific biotopes (rubber plantations, caves, remote villages, primary forests, etc.), as well as their status to insecticide resistance.

These data will allow the Lao MOH to improve vector control strategies.

In addition, a special effort was made to reinforce our ties with the Lao Military Medical Department (LMMD) of the Lao Ministry of Defense. A Memorandum of Understanding (MOU) was signed on 27 May 2014 to allow LMMD staff to carry out joint research projects and to train in IP Laos laboratories.

With regard to training, IP Laos conducted three training courses: A Regional Chikungunya Workshop, a workshop on Taxonomy and Systematics of medically important arthropods for MOH staff from the central and provincial level and a Biosafety and Biosecurity Workshop for MOH staff at the central and provincial level.

IP Laos's junior scientists, working together with their foreign mentors, are becoming more and more involved in the Institutes research programs and are now taking the responsibility to manage these programs. After three years of Scientific Activity, IP Laos is becoming a center of excellence for research and training of Lao scientists/ medical doctors in the area of infectious and vector borne diseases.

Finally, the Lao Ministry of Health and WHO have nominated IP Laos as the front-line laboratory for diagnostic of suspected Ebola cases in Lao PDR. The Ebola public health crisis has prompted IP Laos to upgrade the BSL2+ lab to a BSL3 status with the incorporation of a glove box and autoclave inside the security laboratory. Dr. Marc Grandadam, Head of the Arbovirus and Emerging Viruses

Laboratory is working closely with the Lao MOH and WHO to put into place a failsafe 24 hour Ebola diagnostic platform. IP Laos has also participated in the real-time Ebola simulation exercises with the Lao Ministry of Health and other institutions, such as Wattay Airport, Mittapab Hospital and the National Center for Laboratory and Epidemiology (NCLE).

A decade ago, the idea of the creation of an Institut Pasteur du Laos emerged with the SARS and Avian Influenza H5N1 epidemics. At that time, the Lao Ministry of Health rallied the creation of IP Laos to provide Lao PDR with a research and training facility to deal with highly pathogenic emerging diseases threatening the country and the region. Today, IP Laos stands ready to fulfill this important role and to work with the Ministry of Health and other stakeholders to mitigate the arrival and spread of emerging infectious diseases.

Scientific Activities 2015-2016

Arbovirus and Emerging viral diseases laboratory *Lao-French joint Lab 1*

Since 2012, the Arbovirus and Emerging Viral Diseases Laboratory developed combined field studies to improve knowledge of viral vector-borne diseases in the Lao PDR. Over the last three years, the team was organized around specific projects to help the young Lao scientists and technicians to acquire skills in virology with strong practical applications. In order to expand the capacities of the unit and reinforce the positioning of the laboratory in the public health arena, a new strategy has been developed. The team has now been restructured into three groups that, between them, encompass the different laboratories' areas of responsibility.

Progress in arbovirus surveillance and staff changes afforded opportunities to reorganize the team. A major part of the group is now dedicated to the global surveillance of arboviruses, providing (i) assistance to Vientiane and provincial hospitals for the confirmation of arboviral infections, (ii) updated information and alerts to national and international health authorities, and (iii) training for medical staff in hospitals. A second group focuses its activities on arbovirus ecology. Interactions with entomologists and vertebrate experts were reinforced in order to diversify the fields of investigation to study arbovirus cycles and maintenance in wild environments. A third group is in charge of conducting research on more fundamental aspects of virus–host interactions and to develop new detection or diagnosis tests.

Nevertheless, the capacity of the unit to mobilize sufficient staff to face a major public health crisis in the area of the emergence of unrecorded viral pathogens in the region or re-emergence of endemic viruses on a 24/7 basis has been maintained.

Head of Laboratory: **Marc GRANDADAM, PhD**

Junior Scientists:
Kouxiong SAYTENG, MD
Sompavanh SOMLOR, MD
Thonglakhone XAYBOUNSOU, MD

Technicians:
Chintana LATHAPHASAVANG
Phaithong BOUNMANY
Souksakhone VIENGPHOUTHONG
Sitsana KEOSENHOM
Bounta VONGPHACHANH

Projects

- Arbovirus surveillance
- Arbovirus ecology
- Research/Development

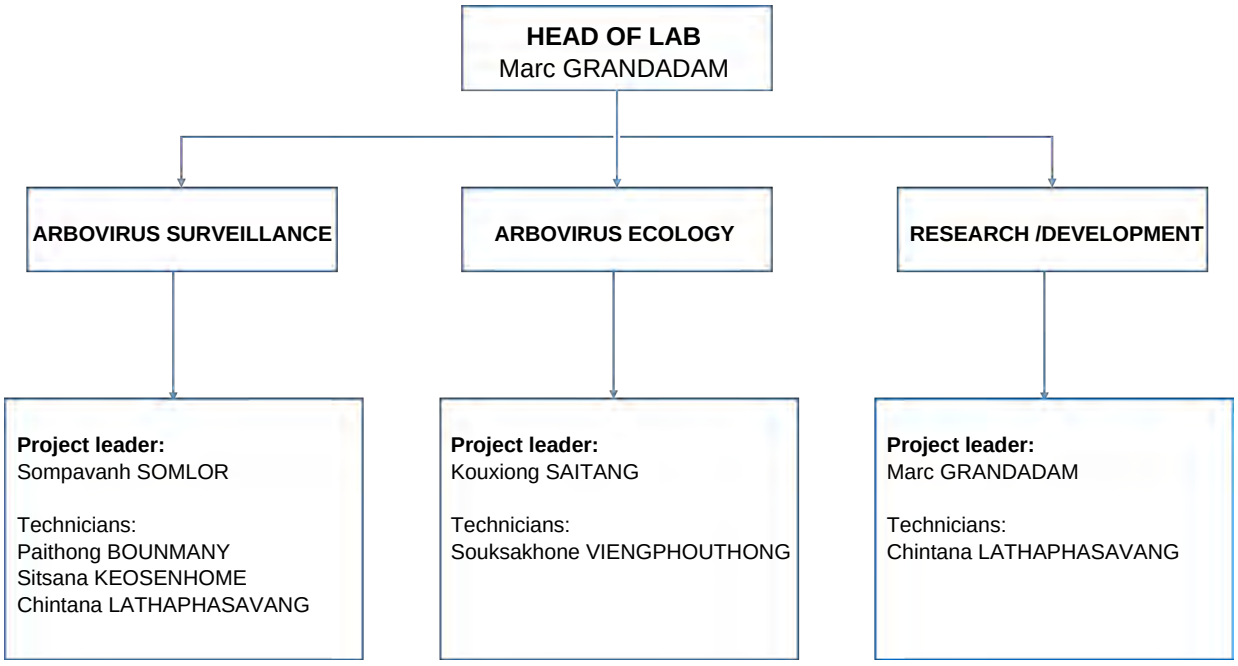


Figure 1: Arbovirus and emerging viral diseases laboratory Lab organigram

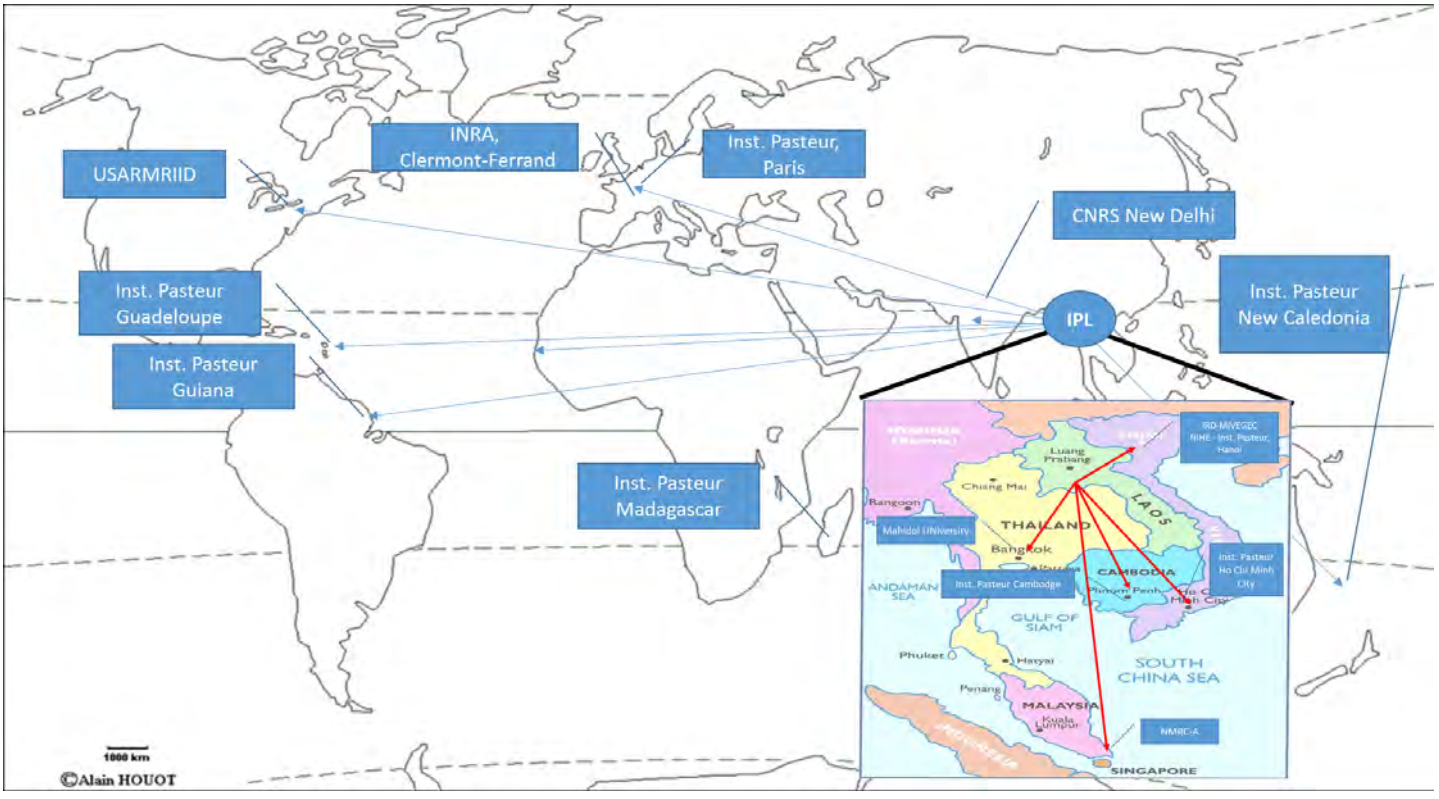


Figure 2: Map of the international interactions 2015

Arbovirus surveillance

Project coordinator: Somphavanh Somlor
Member of staff: Phaithong Bounmany, Sitsana Keosenhome and Chintana Lathaphasavang

Background

Annual dengue fever outbreaks and epidemics have ravaged Laos for decades causing severe mortality and morbidity, as well as significant economic loss. Institut Pasteur of Laos (IP Laos) has been focusing on dengue since its inception. In 2012, IP Laos established the first comprehensive surveillance network in Vientiane capital incorporating 4 civilian hospitals, as well as the military and police hospitals, providing diagnostic confirmation of dengue infections plus viral typing and sequence analysis to better understand the molecular epidemiology of dengue virus circulation in Lao PDR. Data obtained over the four years of surveillance could help the DCDC department of the Ministry of Health of Lao PDR to define a prevention strategy for 2016. As dengue surveillance is not fully coordinates yet (different analysis algorithms; interpretation of tests results; patients' classification…), a clear overview of dengue circulation remains challenging at the country level.

Trends of dengue circulation in Vientiane Capital 2012-2015

Dengue virus is under surveillance by Institut Pasteur du Laos since 2010 in Vientiane Capital with the cooperation of hospitals and private clinics in the city. The team is now a member of the UNITEDENGUE network. The strong mobilization of the clinician allowed collecting data over 4 years. These data permitted to establish the main features of dengue virus circulation. The main outcome of this permanent surveillance system was to evidence an efficient circulation of dengue virus outside the rainy season. This phenomenon has depicted for dengue virus serotype 1 (DENV-1) by the team of the LOMWRU of in rural areas in Lao PDR [Dubot-Peres, 2013].

This "inter rainy circulation" has been documented all over the period studied and all the four dengue serotypes were involved (Figure n°3, 4). Differences in amplitude have been recorded but were more likely linked to environmental variations from year to year rather than on virulence of the dengue viruses.

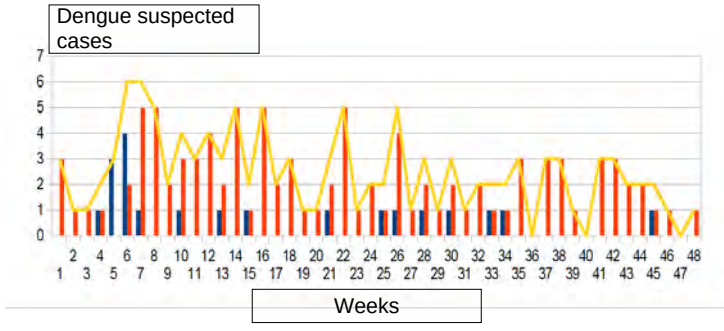


Figure n°3: Dengue surveillance in Vientiane Capital, 2014

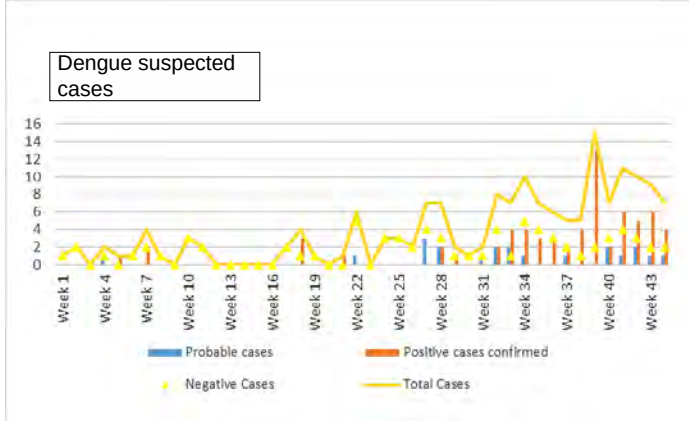


Figure n°4: Dengue surveillance in Vientiane Capital, 2015

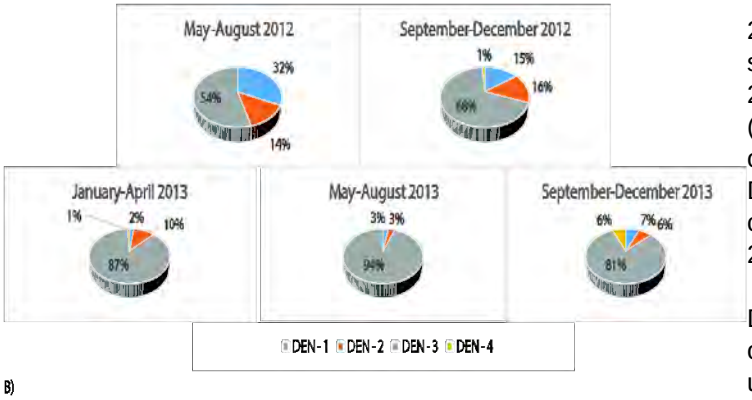
Trends in dengue virus serotypes circulation

Vientiane Capital experienced a continuous but heterogeneous circulation of dengue viruses. Dengue serotype 1 has been at the origin of the 2010 epidemic. Since then, this serotype has been detected permanently excepted in 2014. The same type of circulation has been observed at a lower level for dengue serotype 2.

Dengue virus serotype 3 (re)-emerged in Vientiane Capital in June 2012 and spread out as a predominant serotype until November 2013 and suddenly disappeared (Figure n°5). Dengue 4 has been detected at a very low level in Vientiane suburban areas but surprisingly increased in late 2013 and was at the origin of early cases in the city in early 2014 (Figure n°6).

In 2015, autochthonous cases of DENv-4 cases were again detected in the city. DENv-1 reappeared as the predominant serotype in 2015 (Figure n°7).

Figure n°5 : Dengue serotypes in Vientiane Capital 2012-13



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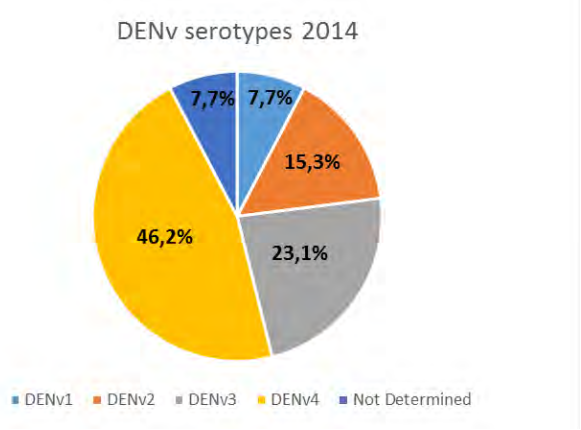


Figure n°6 : Dengue serotypes in Vientiane Capital 2014

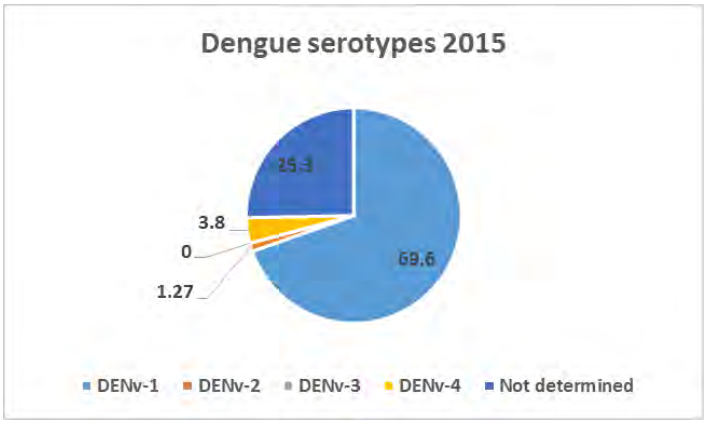


Figure n°7 : Dengue serotypes in Vientiane Capital 2015

Molecular epidemiology help understanding the features of dengue virus circulation in Vientiane Capital. It has been established that DENv-1 strains that circulated between 2010 and 2013 were linked to two independent introduction events whereas DENv-2 strains were maintained over the same period.

The former DENV-3 genotype II strains at the origin of the 2012-13 epidemic displayed strong genetic relations with strains identified in Myanmar between 2005 and 2009 [Lao, 2014]. The emergence of a second DENV-3 genotype (genotype III) has been evidenced during the 2012-13 outbreak. These strains shared high identity levels with DENv strains from Buthan and India highlighting the complexity of the emergence pathways in Vientiane [Lao, 2014].

Dengue 4 strains are under investigation to establish their origin. The 2015 DENv isolates (serotypes 1,2,4) are also under study to determine whether they derived from past autochthonous Lao strains or link to a new introduction event.

These data revealed that, as a possible consequence of the city development and the increasing population exchanges at the regional level, DENv strains circulating in Vientiane Capital are both endemic and imported strains.

Zika virus

Zika virus was first isolated in Uganda in 1947 from a sentinel monkey stationed in the Zika forest. In Asia, the presence of Zika virus has been suspected since the 1960s in India, Malaysia, Northern Vietnam and the Philippines (Figure n°8). The prototype strain of Zika virus in Asia has been obtained in 1981 from a pool of female *Aedes aegypti*

Picture n°1: Vector control around dengue confirmed cases (photo IP Laos)

trapped in a small town in West Central Malaysia. Since than, only few sporadic cases, either autochthonous or imported from Asia in Western countries, have been reported in the Indochinese peninsula. Until now, no evidence of Zika virus in Lao PDR exists. In 2012-13, Lao PDR experienced a huge dengue epidemic but 20 to 50% of the etiologies of dengue like syndromes recorded remain to be determined. We report the detection and the preliminary genetic characterization of Zika virus isolated from patients suspected of dengue. These data evidenced the cocirculation of Zika and dengue virus a local circulation of the virus and its role as an alternative etiology of dengue like syndrom in Laos.

A total of 1353 patients sampled from June 2012 to September 2013 were tested for Zika virus by real-time SybrGreen RT-PCR (Figure n°9). Among them, 18 (1.3%) gave a positive signal by RT-PCR. The virus was diagnosed in 17 Lao residents of Vientiane Capital. None of them reported an stay in a foreign country or in an other province of Lao PDR during the three weeks that preceded symptom onset. The last positive signal was found in an expatriate returning from Thailand. As shown in Figure n°9, Zika virus circulated in Vientiane Capital at least over a period of 15 months. All PCR products displayed a melting temperature (Tm) slightly different from Tm obtained with the Senegalese prototype.

Picture n°2: Training on dengue surveillance and diagnosis in a provincial hospital (photo IP Laos)

Single Zika virus infection represented 61.1% of the cases. Of the 7 (38.9%) dengue-Zika co-infection, six of seven corresponded to Zika-DENV3 co-infections, the last one remains to be determined.

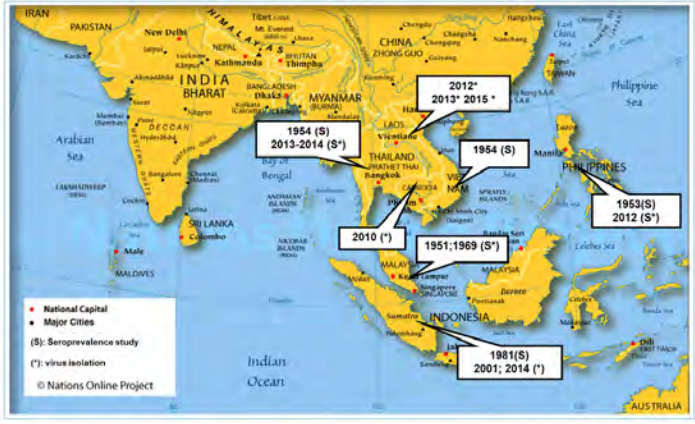


Figure n°8: Zika virus history in Southeast Asia

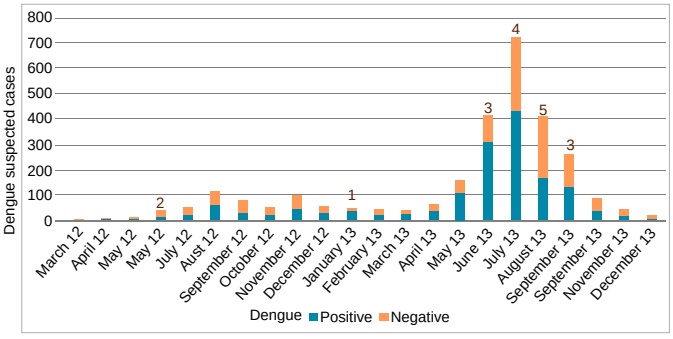
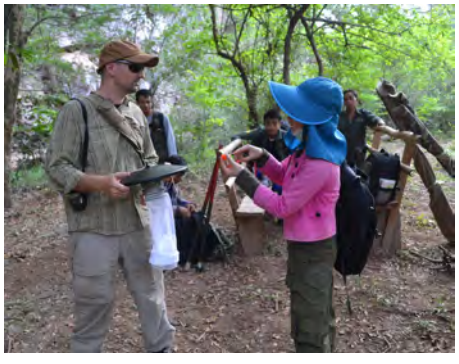


Figure n°9: Distribution of Zika virus cases in Vientiane Capital: 2012-13

Our retrospective study provides the first evidence of Zika virus in Lao PDR. Positive cases were found in patients with a clinical suspicion of a dengue infection. Most of the cases corresponded to single Zika virus infection. Lao PDR faced a continual circulation of dengue virus from 2012 to the end of 2013. Thus, dengue-Zika co-infection could be expected. Such co-infections have already been reported but our series appears as the largest recorded upon now. Interestingly, a co-infection has also been recorded in an imported case from Thailand. This last country also experienced a huge dengue outbreak. Since 2012, an active circulation of Zika virus has been proven in residents of different provinces of Thailand and in tourists returning from this in 2013 and Zika cases were recorded in different provinces. Since 2010, different countries in Southeast Asia reported sporadic cases of Zika virus but no data was available for Lao PDR. These preliminary data shade the light on a new virus in Lao PDR that should now be include in the routine differential diagnosis of dengue like syndroms. Sequence comparison will help understanding how the virus circulated in Vientiane Capital and at the regional level.

Arbovirus ecology

Project coordinator: Kouxiong Sayteng
Member of staff: Souksakhone Viengphouthong



Two main project in the domain of arbovirus cycle description in Lao PDR were achieved in 2015. Both of them were conducted in close collaboration with the medical entomology unit of the Institut Pasteur du Laos.

SandflyMap

Sandflies are known as vectors of viral, protozoan, and bacterial diseases. The sandfly-borne viruses comprise three genera: (1) *Phlebovirus* (Family *Bunyaviridae*), (2) *Vesiculovirus* (Family *Rhabdoviridae*), and (3) *Orbivirus* (Family Reoviridae). The most important groups are the *Phlebovirus genus*, which includes the sandfly fever Sicilian and Toscana viruses, and the Vesiculovirus genus, which includes vesicular stomatitis as well as the Chandipura and Isfahan viruses. The vast majority of sandfly-borne viruses cause only moderately severe diseases in only specific geographical regions, so they are given little attention by physicians and their prevalence is underestimated. The high morbidity is usually among non-native populations such as military personnel and travelers in the endemic regions. A recent review has indicated that sandfly-borne virus diversity in the Mediterranean basin is higher than initially suspected (Maroli, Feliciangeli *et al.* 2013). Furthermore, recent investigations have shown that flavivirus RNA closely related to insect-only *flaviviruses* were detected in sandflies (Moureau, Ninove et al. 2010, Sanchez-Seco, Vazquez et al. 2010). There are high possibilities that sandfly-borne viruses may be circulating in Lao PDR as well as in Southeast Asia, but we lack evidence at present because of their self-limited symptoms within a specific area, the absence of a surveillance system, limited laboratory resources for differential diagnosis and a paucity of scientific interest in sandfly-transmitted viral diseases.

The SAND-Map project involved U.S. Naval Medical Research Center-Asia (NMRC-A) and the Institut Pasteur du Laos (IP-Laos). Its goals were to establish an inventory of sandfly species and their distribution in Laos and to assess their role in pathogen transmission, especially for Phleboviruses.

Five sites were selected for sandfly collection during our study—one in Fueng district, Vientiane province; two in Nakai district, Khammuan province and Korbong village, in Watershed Management and Protection Authority area (WMPA) one in Boualapha district, Khammuan province (Phou Hin Nam Nor National Protected Area (PHNN NPA) and one site in Vientiane capital. From May to June 2015, sandfly collections were performed during the field missions of a parallel project (ResArbo-Coll/ResArbopath see below).

Sampling and identification of sandflies have been performed by the entomology team (refer to the medical entomology annual report).

Pan-phlebovirus RT-PCR screening allowed the identification of viral sequences in 142 of the 1,024 (13.87%) sandfly specimens tested so far. Among them, 59/648 (9.1%) and 83/340 (24.41%) of the positive samples were captured, mainly inside caves, in Khammuan province and Vientiane province respectively. No positive samples were found in Vientiane capital. Almost all positive samples were detected within the *Sergentomyia* and *Phlebotomus* genera and at a lower level in the *Chinius* genus. In the genera *Phlebotomus* *Phlebotomus* and *Sergentomyia*, both female and male were found positive. In Khammuan province, the positive rates of genus *Phlebotomus* and genus *Sergentomyia* were equal at 10% (10.96% and 10.32% respectively). In Vientiane province, the positive rate was 31.36% in the *Sergentomyia* genus and 13.43% in the *Phlebotomus* genus (Table 1).

Table 1: Phlebovirus detection by conventional nested RT-PCR.

Province, district and genus	Total No. of pools	No. of positive pools****	Percent
Khammuan	**648**	**59**	**9.10**
Nakai district	648	59	9.10
Chinius	40	3F	7.50
Corethrella*	62	0	0
Nemopalpus Macquart	3	0	0
Not phlebotominae**	7	0	0
Phlebotomus Rondani & Berté	146	16 (8F, 8M)	10.96
Sergentomyia França & Parrot	378	39 (21F, 18M)	10.32
Unknown***	12	1	8.33
Vientiane capital	**36**	**0**	**0**
Hadsayfong district	19	0	0
Sergentomyia França & Parrot	19	0	0
Naxaythong district	17	0	0
Phlebotomus Rondani & Berté	1	0	0
Sergentomyia França & Parrot	16	0	0
Vientiane province	**340**	**38**	**24.41**
Fueng district	340	83	24.41
Chinius	35	0	0
Not phlebotominae**	1	0	0
Phlebotomus Rondani & Berté	67	9 (2F, 7M)	13.43
Sergentomyia França & Parrot	236	74 (59F, 15M)	31.36
Unknown***	1	0	0
Grand Total	**1024**	**142**	**13.87**

* Corethrella: frog-biting midges, Corethrellidae family,
** Other insects.
*** Unknown. Only head of sandfly present on slide or slide not properly prepared.
**** F=Female, M=Male

Most of the samples positive for phlebovirus sequences were found in specimens collected inside caves from Vientiane province (83/142; 58.45%) and from Khammuan province (59/142 ; 41.55%) whereas no samples from Vientiane capital were found positive. Both females and males within the genera *Phlebotomus* and *Sergentomyia* were positive for viral sequences. Interestingly, in most cases, high infestation rates were found in males nearly equal to those recorded in females. These results suggest that multiple modes of viral transmission are involved in at least these two sandfly genera (i) direct blood feeding for females, (ii) horizontal sexual transmission between females and males, and (iii) vertical transmission to offspring for both males and females. This is the first time that phlebovirus sequences have been detected in sandflies from Lao PDR. Viral species identification is now our main focus. This will be tentatively achieved by viral isolation assays and genomic sequence analysis.

ResArbo

Arboviruses pose major health problems in Southeast Asia and in both rural and urban areas. Intriguingly, sylvatic transmission (i.e. transmission in the wild environment) has been poorly studied in Asia, including for major pathogens such as dengue and chikungunya. Arbovirus transmission often relies on mosquitoes, especially Aedes and Culex. Distribution of the primary vectors of dengue and Chikungunya (i.e. *Aedes aegypti*; *Ae. albopictus*) and of Japanese encephalitis (i.e. *Culex tritaeniorynchus*) is well documented in Southeast Asia.

However, other groups of arthropods have been recognized as primary vectors of arboviral diseases. Ticks are responsible for the transmission of the Tick-Borne Encephalitis virus (TBE) and have been recently recognized as a vector of the newly discovered Severe Fever and *Thrombopenia* virus. Sandflies are well known vectors of *Leishmania spp* parasites but also of *Bluetongue* virus (*Reoviridae*) and neuro-invasive phleboviruses (Toscana; Sandfly Naples). Studies of ticks and sandflies have been neglected in Southeast Asia countries.

Picture n°3: Sample preparation during a Resarbo-Coll project field mission (photo IP Laos)

Picture n°4: Blood sampling on lived vertebrates during Resarbo-Coll project field mission (photo IP Laos)

These gaps in the inventories of both vectors and amplifying/ reservoir vertebrates involved in known and unknown arboviruses may have serious consequences for diagnosis and patient management, epidemiology, and the prevention of human and animal arboviral diseases. Southeast Asia has been considered for decades as an endemic area for dengue, chikungunya, Japanese encephalitis, and numbers of other arboviruses but major questions are still to be answered: i) How do arboviruses maintain themselves in the environment? ii) How do they survive during adverse periods when vectors are not prevalent? Some preliminary data have suggested that animal reservoirs and/or the arthropods themselves could serve as reservoirs. If arboviruses circulate specifically in the wildlife population, the sylvatic cycles could probably switch into the human population in rural areas, then in urban. In order to address these questions, Institut Pasteur du Laos, the National University of Laos, and the Naval Medical Research Center–Asia (NMRC–A) in Singapore proposed a study to assess the temporal–spatial distribution and infection status of arbovirus hosts/vectors and an analysis of putative reservoirs/vectors in karstic and peri-karstic areas of two provinces in Lao PDR

Picture n°5: Sandflies collection in a cave during a Resarbo-Coll project field mission (photo IP Laos

One of the main objectives of the project was to set up a network of experts in order to collect a large spectrum of animal species (including bats, rodents, amphibians, reptiles, hematophagous arthropods, ectoparasites, etc.) that may play a role in the transmission and/or the maintenance of arboviruses in wild environments. Lao limestone karstic areas, drilled by numerous caves, offer ideal study sites to identify such interactions. ResArbo-Coll was coordinated by the Arbovirus ad emerging viral diseases laborartory. This phase of the ResArbo project focused on the collection and identification of the animal species, will provide the foundations of the ResArbo-Path project that aims to evidence arbovirus markers in the biological material and to identify transmission pathways between species.

ResArbo-Path

Vector-borne diseases constitute a significant infectious disease risk for all populations in Laos. However, in Laos, definitive diagnosis is often not available for vector-borne illnesses, so the infectious diseases that are a threat to the population are often not well defined. One of the major biological questions to address is how do vector-borne diseases maintain themselves in nature? How do they survive during adverse periods when vectors are not prevalent? Some preliminary data have suggested that animal reservoirs and/or the arthropods themselves could serve as reservoirs. In order to address these questions and to identify the putative reservoirs of these known and emerging vector-borne pathogens in Laos, Institut Pasteur Laos, the National University of Laos, and the Naval Medical Research Center-Asia (NMRC-A) in Singapore proposed a study to assess the temporal-spatial distribution and infection status of arbovirus hosts/vectors and to analyze putative reservoirs/vectors.

Resarbo-Path constituted the second phase of the ResArbo project and focused on the screening of the samples collected during phase 1 for a selected panel of pathogens.

Blood samples from different vertebrate species were investigated by pan-genus (i.e. alphavirus, flavivirus, and phlebovirus) conventional RT-nested PCR. Among the samples available, whole blood and/or dry blood spots, only whole blood samples were analyzed in order to draw a correlation between RT-PCR screening and viral culture assays. A total of 589 blood samples collected from bats, rodents, reptiles and amphibians were tested with the different RT-PCR systems (Table 2). Of them, 385 (65.4%) were found positive for at least one of the targeted viral gerera Respective proportions for alphavirus, phlebovirus, and flavivirus were 26.2% (154/589), 20.5% (121/589) and 18.7% (110/589) respectively (Table 2).

Amphibians and reptiles presented the highest flavivirus sequence positivity rates with 28.2% (11/39) and 26.7% (16/60) respectively, followed by bats with 17.5% (83/474). Alphavirus sequences were found predominantly in 31.7% of bats (150/474) but also in 18.8% (3/16) of rodents and 1.7% (1/60) of reptiles. All four groups of hosts displayed phlebovirus sequences at significant levels with 18.4% (87/474), 31.3% (5/16), 28.3% (17/60) and 30.8% (12/39) in bats, rodents, reptiles, and amphibians respectively.

Table 2. Arbovirus screening of vertebrate blood samples by RT-PCR

HOST	n	PAN-FLAVI		PAN-ALPHA		PAN-PHLEBO		Multiple infections*			
		Pos (%)	Neg	Pos (%)	Neg	Pos (%)	Neg	F/A	F/P	A/P	F/A/P
Bat	474	83 *(17.5)*	391	150 *(31.6)*	324	87 *(18.4)*	387	15	7	69	7
Rodent	16	0 *(0)*	16	3 *(18.8)*	13	5 *(31.3)*	11	0	0	3	0
Reptile	60	16 *(26.7)*	44	1 *(1.7)*	59	17 *(28.3)*	43	0	2	1	0
Amphibian	39	11 *(28.2)*	28	0 *(0)*	39	12 *(30.8)*	27	0	0	0	0
Total	**589**	**110 *(18.7)***	**479**	**154 *(26.1)***	435	**121*(20.5)***	**468**	**15**	**9**	**73**	**7**

*(n): number; *F/A: flavivirus/alphavirus co-infection; F/P: flavivirus/phlebovirus co-infection; A/P: alphavirus/phlebovirus co-infection; F/A/P: flavivirus/alphavirus/phlebovirus co-infection*

In order to demonstrate actual infection of vertebrates, material from whole blood samples was saved at the beginning of the extraction procedure for immediate inoculation on C6/36 and Vero E6 cell lines. In all, 589 whole blood samples were screened and inoculated on both cell lines.

At this stage, the screening of culture supernatants has been achieved for 452 of the 589 cultures. The 137 remaining passage 1 cultures were stored frozen at −80 °C until RT-PCR analysis. Screening of passage 1 culture supernatants by RT-PCR allowed the detection of flavivirus sequences in 15.9% (72/452) of Vero E6 cultures but from only 0.4% (2/452) of C6/36 culture. Phleboviruses were successfully isolated from 42% (190/452) of the Vero E6 cultures. As shown in Table 4, the number of positive cultures exceeded the number of whole blood samples detected positive for phlebovirus sequences. No alphavirus could be retrieved from either Vero E6 or C6/36 cultures (Table 3).

These results demonstrated that some of the vertebrate species harbor actual arboviral infections. Thus, detection of flavivirus and phlebovirus sequences by RT-PCR on whole blood was not an artifact

Table 3. Screening of vertebrates samples cultures

HOSTS	n	PAN-FLAVI				PAN-ALPHA				PAN-PHLEBO		
		WB	Vero	C6/36	V/C	WB	Vero	C6/36	V/C	WB	Vero	C6/36
Bat	337	75	70*	0	0	134	0	0	0	32	176**	nd
Rodent	16	0	0	0	0	3	0	0	0	5	13	nd
Reptile	60	16	1	1	0	1	0	0	0	17	1(‡)	nd
Amphibian	39	11	1(†)	1	0	0	0	0	0	12	0	nd
TOTAL	**452**	**102**	**72**	**2**	**0**	**138**	**0**	**0**	**0**	**66**	**190**	**-**

n: number of whole blood samples inoculated; WB: indicates the number of whole blood samples positive by RT-PCR ; V/C: positive culture on Vero E6 and C6/36; nd: not done. **21 positive cultures were obtained from whole blood samples screened positive. **17 positive cultures were obtained from whole blood samples screened positive. Five of the 13 rodent samples with positive phlebovirus cultures were obtained from whole blood samples screened positive. (†)(‡) Whole blood screened positive.

At the end of the program, 384 (9%) specimens of the 4232 sandflies could be tested for the presence of *phlebovirus* sequences because of the time taken for the specimens identification and preparation processes. Among these samples, 11 (2.9%) were found positive. The 11 positive samples were distributed respectively in 4 (36.4%) and 7 (63.6%) of sandflies belonging to the *Phlebotomus* (ref: Rondani & Berté) and *Sergentomyia* (ref: França & Parrot) genera (Table 4). Most of the positive specimens were found in females but, in both genera, infected males were also found.

Table 4: Phleboviruses detected in sandfly genera

GENERA	POSITIVE (%)	FEMALE	MALE
Sergentomyia França & Parrot	4 *(36.4)*	3	1
Phlebotomus Rondani & Berté	7 *(63.6)*	6	1
TOTAL	**11 *(100)***	**9**	**2**

Virologic investigation of vertebrate and arthropod specimens collected during two field missions in Vientiane and Khammuan Provinces provided information on arbovirus geographic distribution and possible inter-species relations that may contribute to arbovirus maintenance in wild environments.

Phlebovirus sequences were evidenced in all groups of vertebrates investigated, which comprised bats, rodents, reptiles, and amphibians. Moreover, the presence at the same time and in the same areas of the main recognized arthropod vector of phleboviruses, i.e. sandflies, strongly supports a local transmission of phleboviruses. Interestingly, the presence of viral sequences in males sheds light on possible complex mechanisms of phlebovirus maintenance that may include vertical and horizontal transmission in sandfly populations. The role of the different vertebrate species in this(ese) cycle(s) remains to be determined. Indeed, no conclusion could be drawn at this stage on the significance of the viral sequences detected to determine if vertebrates participate as a reservoir, amplifying host, or dead-end host. However, successful viral isolations from bats, rodents, and reptiles demonstrate that a large spectrum of vertebrates is supporting phlebovirus replication. Sequence data are now needed to identify the viral species and establish the links between arthropods and vertebrates.

Most members within the flavivirus and alphavirus genera are known to be transmitted by mosquitoes. The arthropod collection strategy (type of trap, collection sites) was more adapted to sandflies. This explains, at least in part, the lack of mosquito samples. Nevertheless, evidence of the presence of flavivirus and alphavirus has been established by sequence detection and/or viral isolation.

Flavivirus infection occurred with bats, reptiles, and amphibians but was not evidenced in rodents. Alphavirus detection was also positive in all vertebrate groups except amphibians. However, as the number of specimens was limited, these results do not exclude a possible infection of rodents by flavivirus and amphibians by alphavirus.

Altogether, this exhaustive inventory of the caves' fauna allows us to speculate about a specific transmission cycle of phleboviruses in the caves. The absence of mosquitoes inside the caves supports the hypothesis of more complex cycles for flavivirus and alphavirus, within which transmission of viruses to cave-dwelling vertebrates occurs during animal displacement outside the caves. For instance, *Rattus rattus* is a rodent species which often shares human habitats in the villages located near the caves where environmental conditions are more adapted to the proliferation of mosquito species. This does not exclude the existence of alternative vectors for flavivirus and alphavirus. Indeed, recent studies evidenced flavivirus sequences in sandflies and our team found alphavirus sequences in ticks *(refer to TickMap project report).* Future investigations planned on ectoparasites collected from vertebrates may bring some answers.

Potential dual and triple infections involving the arboviral genera tested have been evidenced in different vertebrates. Among the bat specimens, 3.2% were co-infected by flavivirus and alphavirus, 14.6% were positive for alphavirus and phlebovirus sequences and 1.5% displayed sequences of the three viral genera. This phenomenon was not limited to bats. Even if the specimen number was limited for rodents and reptiles, different patterns of multiple infections could be evidenced. These last results suggest that the vectors transmitting the viruses may have a wide feeding pattern. Sequence analysis will help in exploring this question.

Viral sequences belonging to the three genera were detected in significantly distant study sites. These data support a high viral biodiversity in each province and possibly at the country level. It is not yet known whether the members of the different viral genera present in the two provinces are identical, represent genetic variants of the same species, or are independent species. An extended sequencing program is now needed to establish this degree of biodiversity and to draw up hypotheses on the distribution of the viruses.

This one-year program allowed the gathering of preliminary results on viral biodiversity in two provinces of the Lao PDR. The different sites can be considered as hot spots in terms of rates of infection, diversity of infected vertebrates, and diversity of viral genera circulating at the same time in the same place. As only a unique field mission could be organized for each site, it is not possible to determine the

influence of seasonality. However, as the missions took place at the end of the dry season, it can be expected that infection rates and both vertebrate and arthropod diversity may increase temporarily during the rainy season. Cave environments explored during this project offer optimal conditions for virus spillover as illustrated by the infection rates in animal populations. Our study sites in wild environments but located at the limit of human-occupied areas could be used as sentinel sites to assess the risk of viral emergence in human populations.

Research and development

Project coordinator: Marc Grandadam
Member of staff: Chintana Lathaphasavang

The availability of serological tests remains a critical issue to face emerging arbovirus. In 2014, the A&EVD laboratory launched preliminary developments to set up MAC-ELISA tests that do not require highly specific reagents such as antibodies from hyper-immune ascetic fluids.

A first prototype for chikungunya virus IgM detection is now under validation through a multicentric study (Valoexpress MACH-2). Encouraging preliminary results led to apply the technology to new emerging arboviruses in Lao PDR. Using reference strains (Zika) or primary isolates obtained from fields specimens (phlebovirus), antigen were produced on a large scale to prepare prototypes for serological kits (IgM and IgG). Zika ELISAs will be validated in 2016 on a reference panel of sera provided by the Institut Pasteur de Nouvelle Calédonie. The phlebovirus tests have been utilized to rescreen a collection of sera collected in groups of the Lao population living in the geographic area from which the phlebovirus were isolated.

Emerging viral diseases/Response to public health threats

In September 2015, the A&EVD laboratory was approached by the Lao Ministry of Health to reinforce the national laboratory capacity to face a possible outbreak of MERS-CoV. A procedure adapted from the Ebola alert system set up in 2014 was adopted. Methods were obtained with the collaboration of CIBU (Institut Pasteur, Paris) and validated by participation in an international proficiency test organized by the World Health Organization

Support activities

The A&EVD laboratory supported different Institutes within the Pasteur Network. Table 5 summarizes biological material transfers in 2015.

Table 5: Biological material transfer activity, 2014–2015

Consignee	Biological material
Institut Pasteur Ho Chi Minh City	Anti-Chikungunya virus IgM MAC-ELISA prototype kit
Institut Pasteur de Nouvelle Calédonie	Anti-Chikungunya virus IgM MAC-ELISA prototype kit
Institut Pasteur de la Guyane	Anti-Chikungunya virus IgM MAC-ELISA prototype kit

Education activities

- Information on dengue surveillance and Rapid diagnosic tests: Attapeu Provincial Hospital, Saravanh Provincial Hospital (6 days, August 2015).

- Dengue virus diagnosis: Training sessions for staff and students in Vientiane Capital Hospitals (Mitthapad; 5 April; Setthathirat; Children hospital; half a day per hospital).
- Diagnostic de la dengue / infection à virus chikungunya: Institut de la Francophonie pour la Médecine Tropicale (1/2 day, Decembre 2015).

Training of students

Table 6: Synthesis of students welcomed in 2015.

Names	Degree	Institution	Time period	Subject
Bounmy OUTHAYAVONG	Lab technician	Epidemiology and Prevention centre of the Lao Army	May-July 2015	General medical virology and arbovirology
Thonglakhone XAYBOUNSOU	Master 2	Institut de la Francophonie pour la médecine tropicale, Vientiane, Lao PDR	April-August 2015	Kinetic of dengue virus viremia in Lao patients
Van Duong NGUYEN	Master 2	Institut de la Francophonie pour la médecine tropicale, Vientiane, Lao PDR	April-August 2015	Prevalence of chikungunya virus infection in Southern Vietnam

- (Marc Grandadam) Member of the jury of thesis defense of doctorate: Anne-Claire ANDRIES. Phnom Penh, December 17th 2015.
- Biosafety/Biosecurity course (Phase 1 in Novenber 2014; phase 2 onsite visit to assess the capacity of the trainees to be trainers; June 2015).

Scientific communications

Poster:

Kouxiong SAYTENG; Malayvanh Lao; Souksakhone Viengphouthong; Paul T. Brey and Marc Grandadam First detection of Zika virus in Lao PDR. International Scientific Symposium - Institut Pasteur International Network. Paris, October 14th-16th, 2015.

Scientific articles:

1. Li MY, Grandadam M, Kwok K, Lagache T, Siu YL, Zhang JS, Sayteng K, Kudelko M, Qin CF, Olivo-Marin JC, Bruzzone R, Wang PG. KDEL Receptors Assist Dengue Virus Exit from the Endoplasmic Reticulum. Cell Rep. 2015 Mar 3. pii: S2211-1247(15)00167-9. doi: 10.1016/j.celrep.2015.02.021.
2. Okabayashi T, Sasaki T, Masrinoul P, Chantawat N, Yoksan S, Nitatpattana N, Chusri S, Morales Vargas RE, Grandadam M, Brey PT, Soegijanto S, Mulyantno KC, Churrotin S, Kotaki T, Faye O, Faye O, Sow A, Sall AA, Puiprom O, Chaichana P, Kurosu T, Kato S, Kosaka M, Ramasoota P, Ikuta K. Detection of chikungunya virus antigen by a novel rapid immunochromatographic test. J Clin Microbiol. 2015 Feb;53(2):382-8. doi: 10.1128/JCM.02033-14. Epub 2014 Nov 19.
3. Vanessa M. Monteil, Marianne Maquart, Valérie Caro, Marie-Christine Jaffar-Bandjee, Marc Grandadam, Isabelle Leparc-Goffart. Circulation of Dengue virus type 3 genotype III in Africa since 2008. Submitted to Infection Genetics and Evolution.
4. Andrew J Taylor, Khamsing Vongphayloth, Malavanh Vongsouvath, Marc Grandadam, Paul T. Brey, Paul Newton, Ian W. Sutherland, Sabine Dittrich. Novel tick-borne bacterial pathogens in the Lao PDR – implications for human disease. Submitted to Emerging infectious Diseases.

Other documents:

- Dengue virus surveillance in Vientiane Capital 2012-15. Trends and perspectives. Lao Ministry of Health. Department of Communicable Diseases Control. November 2015.
- General security Manual of the Institut Pasteur du Laos

Medical Entomology & Biology of Disease Vectors Laboratory *Lao-French joint Lab 2*

The main objective of our lab is to study the biology and ecology of arthropod vectors (mosquitoes, sandflies, ticks, snails, etc.), as well as the transmission cycles of the viruses, parasites and other microbial pathogens they transmit. Furthermore, we are working on ways to mitigate vector borne disease transmission in Lao PDR via vector control training programs.

Head of Laboratory: **Dr. Paul BREY, PhD**

Scientists:
Sébastien MARCOMBE, PhD
Khamsing VONGPHAILOTH, MD

Junior Scientists:
Phoutmany THAMMAVONG, MD
Julie BOBICHON, MS

Technicians:
Boutsady SOMPHONG
Somsanit CHONEPHESARATH
Nothasine PHOMMAVANH
Kaithong Lakeomany
Lae THUTKHIN, MS
Khanthalak KHOUNSOMBAT, BS

PhD. Student:
Julie anne TANGENA, MS

Projects

- Malaria vectors in Lao People's Democratic Republic and Thailand and capacity building in medical entomology (MALVEC)
- Risk Of Vector-Borne Diseases In Relation To Rubber Plantations In Lao PDR (ECOMORE)
- Risk of Vector-borne Diseases in Relation to Rubber Plantations in Côte d'Ivoire as compared to Lao PDR
- Vector mapping, characterization of insecticide resistance of Aedes populations, and entomology capacity development in Lao PDR (ARBOVEC)
- Efficacy of alternative larvicides for dengue vector control in semi-field trial

Malaria vectors in Lao People's Democratic Republic and Thailand and capacity building in medical entomology (MALVEC)

Project coordinator: Dr. Sébastien Marcombe and Dr. Paul BREY
Member of staff: Julie BOBICHON (Junior scientist), Boutsady SOMPHONG (Technician) and Nothasine PHOMMAVANH (Technician)

Background

In Lao PDR, a recent national survey on the distribution of malaria showed that 65% of the population was still living in transmission areas (Jorgensen *et al*., 2010). This study also showed the predominance of *Plasmodium falciparum* particularly in the southern part of the country associated with a high risk of transmission.

In 2004, an entomological survey showed that *Anopheles dirus* was an important malaria vector despite its low density and that the role of *An. minimus* in the transmission varied over time and space (Trung *et al*., 2004). However, the successive appearance in tropical forest areas of *An. minimus* during the dry season and *An. dirus s.s.* during the second part of the rainy season allows a sustainable malaria transmission. More worrying, the recent environmental modifications linked to agriculture and forestry culture (e.g. rubber plantations) may change the status of several vectors, secondary and major, by giving them appropriate ecological conditions to thrive (Osbomer *et al*., 2007). Insecticide bioassays showed that *An. minimus* was resistant to pyrethroids in northern Vietnam and Thailand and *An. epiroticus* was resistant to DDT and pyrethroids in Cambodia and southern Vietnam (Van Bortel *et al*., 2008). It is possible that the use of agricultural insecticides may be at the origin of the selection of these resistances and so constituting a danger for the implementation of effective vector control strategies. Unfortunately, there is a paucity of data available on the insecticide resistance of the main malaria vectors in Lao PDR. The "hot-spots" of transmission being located in border zones (Thailand, Cambodia, Vietnam...), there is an important risk of dispersal of the population of vectors and the resistances in the surrounding areas. In Lao PDR no data are available regarding the impact of agriculture pesticides on the resistance selection. The only available means of control of the transmission is the use of pyrethroid treated bed-nets, but in Laos, 30 to 50% of the people at risk sleep under treated bed-nets. We do not know if the malaria vectors from Thailand and Lao PDR are endophagic or exophagic. For example, *An. dirus* is known to be exophagic, biting people at twilight at a time of day when that is not protected by treated bed-nets. Hence, it is necessary to understand the vectors biology in Lao PDR and Thailand to adapt the vector control strategies.

The risk of distribution of the insecticide resistances of vectors in South-East Asia represents a serious threat to the good results recorded these last years in the control of malaria. It is urgent to identify the distribution, the levels and the mechanisms of resistance of the vectors in the lower Mekong countries with the aim of helping the health authorities to develop more effective strategies of prevention and control of the disease.

Objectives and outcomes

This project has 4 fundamental objectives:

- Evaluation of vectors bionomics and distribution and their role in malaria transmission
- Evaluation of the levels, types and mechanisms of insecticide resistance
- Evaluation of the impact of environmental factors on vector dynamic and resistance selection
- Capacity building in medical entomology in Lao PDR

Expected outcomes:

- Set up a comprehensive map representing the "hot spots" for malaria transmission in Lao PDR and Thailand (border area)
- Generate an Insecticide Resistance database in the main malaria vectors
- Address the dynamics and gene flows between malaria vectors populations
- Guide public health authorities in the design and implementation of Insecticide Resistant Management strategies
- Capacity strengthening of Lao and Thai students in medical entomology and vector control

Methods

Field sites

The study took place in 10 provinces distributed throughout Lao PDR and 5 districts in Ubon Ratchathani province in Thailand (Figure 1). This enables to have a global vision of the situation in the country. CMPE already have an important collaboration network with all the health district departments, which is a fundamental parameter for the success of the project.

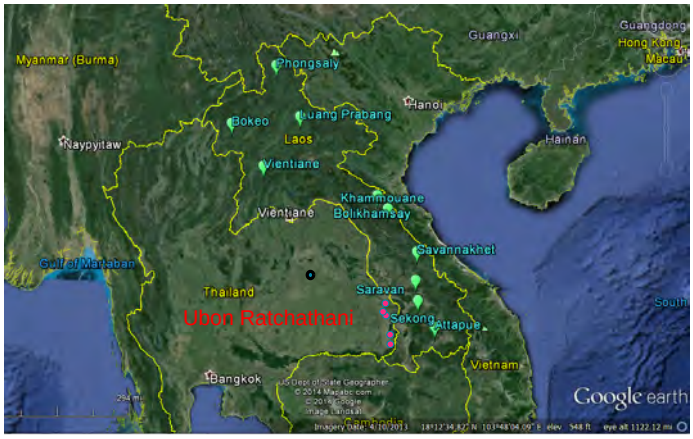


Figure 1. Sampling locations in Lao PDR

Mosquito collection

Every village was divided into four zones from a central axis to select at random 1 house by zone. The selected houses were distant from each others of at least 30 meters. For every house a collector was placed inside and another one outside from 6:00 pm in the evening till 6:00 am in the morning on 4 consecutive nights. Mosquitoes were collected with glass tubes (Figure 2). Mosquitoes were also collected overnight with the cow/buffalo bait collection method. A long mosquito net was disposed around the animal and adult mosquitoes were collected on it (Figure 3).

Figure 2. Human landing catching

Figure 3. Buffalo bait collection

Mosquito identification

The mosquitoes collected were morphologically identified to the species or complex using microscopes and following the identification keys (Medical Important Anophelines of Southeast Asia) (Figure 4). The Anopheles collected were then separated by species for the insecticide resistance tests.

Figure 4. Field laboratory in Vientiane province

Insecticide resistance

Insecticide bioassay (tube tests) were performed following WHO protocols to measure the insecticide susceptibility of the different mosquito species collected (WHO 2013). Adult female were exposed to DDT (4%), deltamethrin (0.05%), and permethrin (0.75%), the main insecticides used in public health in Lao PDR.

Figure 5. Insecticide resistance test.

Results 2015

Mosquito abundance and diversity

Figure 6 shows the number of *Anopheles* collected in the 10 provinces during the dry and rainy season. More mosquitoes were collected during the rainy season and Luang Prabang, Vientiane and Bokeo province showed the highest number of *Anopheles sp*. collected. Figure 7 (A and B) shows the number of *Anopheles sp*. collected on human and cow during the dry and wet season 2015. During the dry season, a total of 1317 *Anopheles sp*. was collected and among them 28% were collected on human. During the rainy season, 5150 *Anopheles sp*. were collected and among them 30% on human. Figure 7 (C and D) shows the *Anopheles sp.* collected from human landing catching. During the dry season, 34% were collected indoor and during the rainy season 47% were collected indoor.

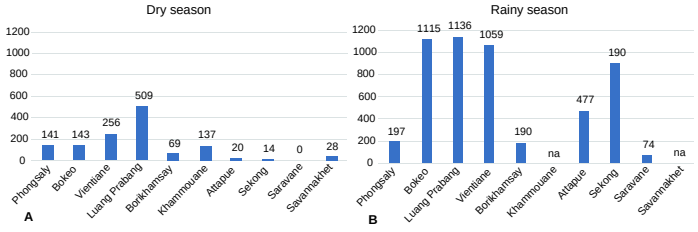


Figure 6. Total number of *Anopheles sp.* collected from Human Landing Catching in the ten provinces during dry and rainy season 2015 (A and B).

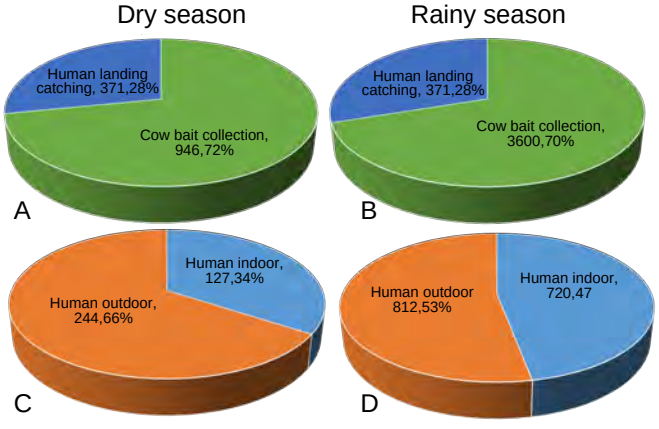


Figure 7. Total number of *Anopheles spp.* collected from human landing catching and cow bait collection during the dry and rainy season 2015 (A & B); inside and outside the house (C & D).

During the dry season, 13 different species of *Anopheles sp.* were collected on human (Figure 8). The two most abundant species were *Anopheles minimus*, *An. maculatus* which are the primary malaria vectors in the Lao PDR (primary vectors, n=55 and n=170 respectively). During the rainy season, 18 different species of *Anopheles sp*. were collected on human (Figure 9). The most abundant species was *An. minimus*, representing 29% of the total of mosquito collected on human (n-453).

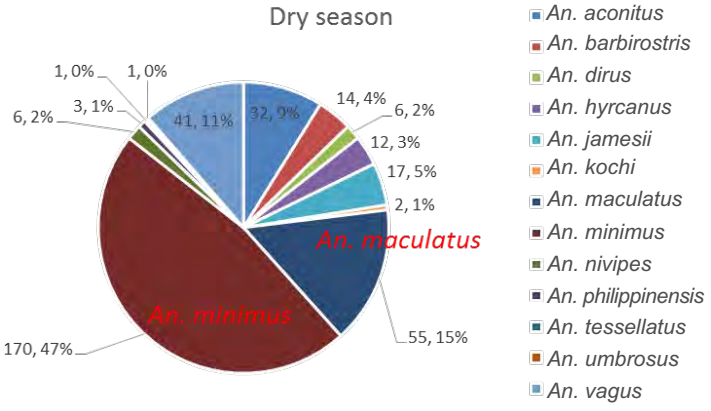


Figure 8. Abundance and proportion of *Anopheles spp.* collected from HLC during the dry season 2015.

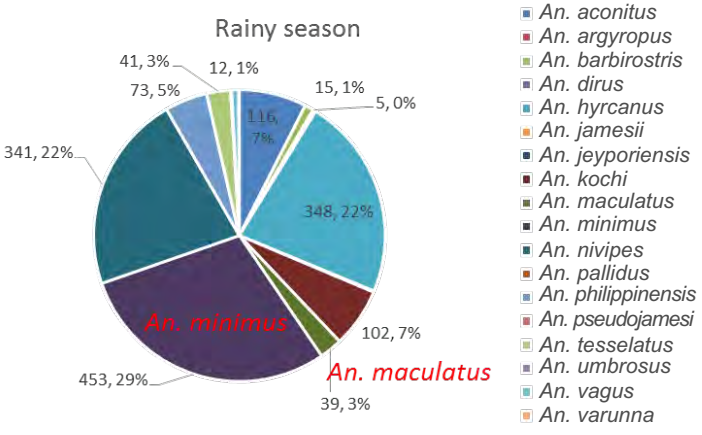


Figure 9. Abundance and proportion of *Anopheles spp.* collected from HLC during the rainy season 2015, Lao PDR.

Human biting rates

Figure 10 shows the human biting rates (number of *Anopheles* collected per man per night) in the 10 provinces in 2015. During both dry and wet season Vientiane and Luang Prabang provinces collection presented the highest HBR. HBRs were lower in average during the dry season compared to the rainy season.

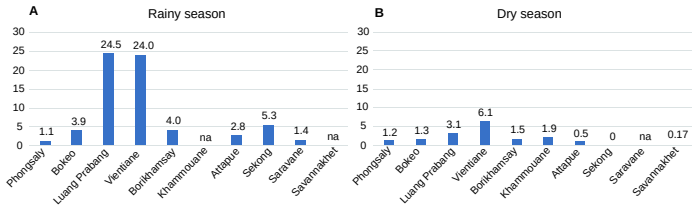


Figure 10. Human biting rates during the rainy (A) and dry (B) season 2015 in the 10 provinces. HBR is the number *Anopheles sp*. collected per human per night.

Insecticide resistance

The results are shown figure 11. Several species showed resistance or a reduced susceptibility to the insecticide tested. Among the primary vector tested, *Anopheles maculatus* showed a reduced susceptibility to permethrin and DDT in Phongsaly while *An. minimus* also showed a reduced susceptibility to DDT in Luang Prabang. The secondary malaria vector An. nivipes showed resistance to the 3 insecticides tested.

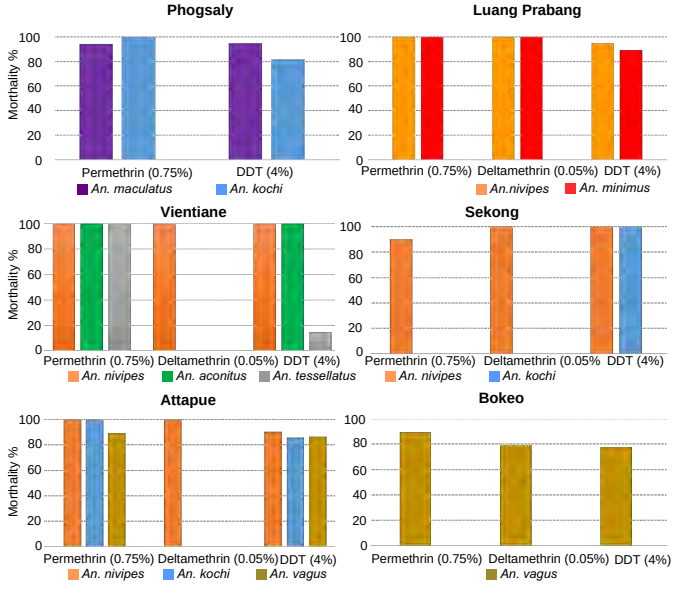


Figure 11. Resistance status of *Anopheles sp*. against permethrin, deltamethrin and DDT, Lao PDR 2015.

Discussion and perspectives

The results from Lao PDR showed that *An. minimus* and *An. maculatus*, primary vectors of malaria, and several secondary vectors, are biting humans constantly during the night both indoors and outdoors. This emphasizes the need for use of bed nets when people are sleeping and personal protection when people are outside. However insecticide resistance tests showed that several *Anopheles* species are resistant to DDT and to pyrethroids (used for bed nets coating) in several provinces of the Lao PDR, emphasizing the need for a constant monitoring of insecticide resistance in malaria vectors in the area.

Molecular work to identify the sibling species of the different group/complex of *Anopheles* species started in 2015. Plasmodium detection in these mosquitoes will also be implemented. Furthermore the possible mechanism involved in insecticide resistance, metabolic and target mutation, will be researched.

Partners

- National Center of Malariology, Parasitology and Entomology (CMPE), Vientiane, Lao PDR
- Institut de Recherche pour le Développement (IRD) IRD-MIVEGEC, IRD UMR-MD3, Bangkok Thailand
- Kasetsart University, Department of Entomology, Bangkok, Thailand
- Institut de Médecine Tropicale d'Anvers (IMTA), Belgium
- University of Life Sciences (ULS), Oslo, Norway
- Bureau of Vector Born Diseases (BVBD), Ministry of Health, Thailand
- World Health Organization (WHO)

Financial support

The MALVEC project was initiated thanks to the research grant from the Initiative 5% of the Global Funds to fight Aids, Tuberculosis and Malaria and Expertise France

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Van Bortel W, Trung HD, Thuan le K, *et al*: The insecticide resistance status of malaria vectors in the Mekong region. *Malar J* 2008, 7:102.

Risk Of Vector-Borne Diseases In Relation To Rubber Plantations In Lao PDR

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ECOnomic development, ECOsystem MOdifications, and emerging infectious diseases Risk Evaluation

Background

South-East Asia (SEA) is experiencing unprecedented economic growth which is transforming land-use on a massive scale in the region. An increasing number of studies show that these land use changes are a major force behind the surge of emerging infectious diseases. In recent years epidemics of SARS, Nipah virus, cholera, malaria, dengue and Japanese encephalitis have been documented [1-4]. Vector-borne diseases are emerging and increasing in areas where ecological disruption occurs, driven by population growth, land use change, urbanization, agricultural reforming, and human migration [5-9].

China is at the heart of the economic growth in the region and its economy has been growing since 1978 at a rate of ± 9 % per year, approximately three times the global average [10]. The economy of the region has been growing since the major economic reforms, with gross domestic product per capita increasing from 1,339 USD in 1978 to 10,540 USD in 2010 [11]. China officially became the second largest economy in February 2011 and it is likely to surpass the United States to become the largest economy in 2020 [12].

All SEA countries are benefitting from the Chinese growth [13, 14]. China imported 341 billion US dollars' worth of products from SEA in 2009, which was nearly half of China's total import [13].

One of the clear examples of the strong economic ties between China and SEA is the rubber industry which supports the rapidly expanding Chinese car industry. In China 18.5 million cars were manufactured in 2010 for which an estimated 3.85 million tons (34 % of the global demand) of rubber was needed, mainly for tyres [15, 16]. Rubber consumption has been increasing 4% annually in the last decades with 42% of this being produced naturally from plantations [15, 17]. Natural rubber production could not keep up with the increased demand and caused prices to reach record highs in 2011 after the natural rubber stock of the world reached a negative 367,000 tonnes in 2010 [18-21]. In Lao PDR this meant latex prices increased from 3,000 kip/kg (0.37 USD) in 2002 to an inflated 15,000 kip/kg (1.87 USD) in 2011 [22]. The increase in price of natural rubber from 2002 onwards encouraged many more to invest in rubber plantations. In Viet Nam the total area for rubber plantation rose from 77,000 ha in 1976 to 465,000 ha in 2005, with land designated for rubber plantations close to 700,000 ha [23]. Presently an estimated 9.161 million ha of rubber plantations exists in SEA [24]. Rubber plantations are largely found in Thailand, Indonesia and Malaysia, together accounting for 72% of global natural rubber production. Thailand in 2009 had 2.8 million ha of rubber growing region with almost 5 million tonnes of natural rubber produced every year [25, 26]. South East Asia supplies 97 % of the world's natural rubber [27].

The high natural rubber production after the peak of investment together with the global recession from 2008-2010 created an excess of natural rubber and natural rubber prices have slumped since 2011 [28, 29]. In December 2013 natural rubber production growth was 3% whilst it's utilization shrank [30]. Natural rubber prices in Lao PDR dropped from 15,000 kip (1.87 USD) in 2011 to 7,000-8,000 kip (0.75-0.87 USD) per kg in 2014 [22]. In 2013 the world natural rubber production was 12,042,000 tonnes with 687,000 tonnes of rubber overproduced, resulting in 3,006,000 tonnes in stock [31]. Consequently from the beginning of 2014 natural rubber prices have dropped more than 20% [32, 33]. The decline in the price of rubber is concerning many natural rubber producers and have even led to protests in Thailand [34, 35]. Although difficult to predict, the International Rubber study Group expects demand for natural rubber to increase until at least 2020 [15, 21].

It takes about five to eight years for the trees to mature, from when the trees can be tapped for rubber for up to 30 years. The whitish latex used for rubber production is present outside the phloem in latex vessels of the bark. These vessels are curved at a 30° angle up the tree in a right-handed spiral. This spiral makes tapping latex very difficult and requires a certain skill. A sequence of thin slices of bark are cut without damaging the growing layer (Figure 1). Every worker is able to tap between 300-450 trees per night equivalent to 1ha of rubber plantation.

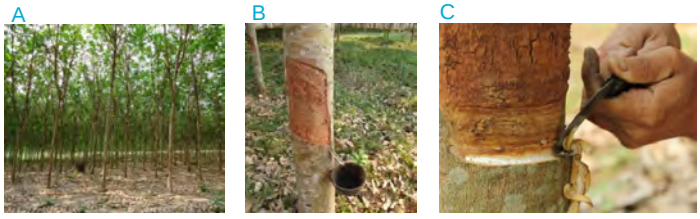


Figure 1: Rubber plantation in Luang Prabang province, Lao PDR (A) Rubber plantation (B) Rubber tree with collecting cup (C) Cutting of rubber tree for tapping

Lao PDR has seen a high increase in rubber plantations (Table 1), where relatively low number of plantations are present compared to neighbouring countries [17, 27]. This is a new kind of mass farming not seen in Lao before. There are two different kinds of rubber plantations in Lao PDR; small scale Lao owned rubber plantations and industrial scale plantations. After 2015 the area of rubber plantations will continue growing with currently 342,400ha of land designated for rubber plantations[36].

Table 1: Mature rubber plantations in Lao PDR (hectare)

Year	Mature rubber plantations in Lao PDR (hectare)
2010	900
2011	6,900
2013	35,960
2015	147,500

In the next decade an estimated 6 million people will be working as tappers on rubber plantations in SEA, which highlights the importance of understanding the exposure risk to mosquito-borne diseases for these workers. Currently there is a paucity of information on vector-borne disease risk in rubber plantations despite the fact malaria epidemics have been linked to rubber plantations in SEA for over a century, , as have outbreaks of dengue and the presence of lymphatic filariasis [37-41]. Data on mosquitoes, vector-borne disease epidemiology and human movement are often difficult to collect for rubber plantation areas with low quality surveillance, unregistered migration, insufficient capacity and difficult to reach areas.

The range of vectors found in rubber plantations is likely to be broad and similar throughout Asia. In one study in India vectors of dengue, chikungunya, Japanese encephalitis, malaria and filariasis were found in rubber plantations [42]. Rubber plantations are unnatural forests with generally higher humidity and lower temperatures than other non-tree crops. This is a habitat that some important vector species like *Anopheles dirus* and *Aedes albopictus* prefer [40, 43-46]. These extremely anthropophilic mosquitoes thrive in these unnatural forested areas of SEA as there are sufficient larval breeding sites, high humidity, stable temperatures, high rainfall and frequent contact with primates and people.

Seasonal workers at the plantations could create a whole new dynamic to the vector-borne diseases in the plantations. Currently 37,500 people work on rubber plantations in Lao PDR of which 95.4% are Lao [36]. The increase in rubber plantations in the coming decade is expected to create work for another 177,700 people. This will increase the need for seasonal workers from other areas. These temporary workers may not have immunity against the local diseases and are more likely to develop serious adverse effects. Additionally, these workers can spread the disease when they travel to areas where the vectors are already established. Even more worrying is the possibility to introduce a drug resistant strain, like ACT resistance, in an area by either returning home with a resistant strain or transporting the resistant strain to the place of work. It is suggested that the presence of high numbers of different vector mosquitoes combined with the increase in seasonal rubber workers and their high exposure to the vector mosquitoes is creating a ‘perfect storm’ in and around rubber plantations for future disease outbreaks

Rationale

The area of land cultivated for rubber is expanding rapidly in Lao PDR. We anticipate that the changes in ecology from primary and secondary rainforest, to rubber cultivation and the maturation of these rubber trees is likely to result in an altered risk from vector-borne diseases; predominantly malaria, dengue and chikungunya. It is envisaged that this study will provide an opportunity to understand the vector ecology in rubber plantations and be able to advise organizations on how to decrease vector-borne disease incidence. This study will be of relevance to public health

workers, governments and those working in the rubber industries of Lao PDR and other countries in SEA.

All methods were approved by the Lao Ethics committee and Durham University ethics committee. The comparison study was also discussed with the CORC-ethics committee of Institut Pasteur and approved.

Overall goal: To assess the potential risk of vector-borne disease infections arising in rubber plantations

- Recognize possible vectors and the diseases they carry in the rubber plantations
- Identify at which times rubber workers are exposed to vectors
- Write public health advice for health workers, governments, and those working in the rubber industries of Lao PDR and other countries in South-East Asia how to decrease and/or keep vector-borne disease incidence low

Methods

During 2015 the ECOMORE team has been busy conducting the final fieldwork and analysing the results of previously conducted fieldwork. We have been focussing on the publication of our results in scientific journals, presenting our work at different national and international conferences, sharing our results and recommendations with stakeholders. Most importantly we have been working closely with the Lao government to include our recommendations in policy.

Longitudinal study on mosquito ecology in rubber plantation

To understand the ecology of the mosquitoes in the rubber plantations, we compared the mosquito number and diversity of the plantation with the forest and village for one year. We collected mosquitoes in the different habitats using the human-baited double net trap (Figure 2), which does not expose participants to mosquitoes. In 2013-2014 mosquitoes were collected in three study areas located in bordering Nane and Xieng Ngeun district (Luang Prabang province) where the following four habitats were present; immature rubber plantation, mature rubber plantation, village and forest. In 2013 we sampled mosquitoes with three participants every hour for two days and two nights in the three study areas every month from July to November. In 2014 this was carried out every two months from January to July. A total of 3,888 hours of data was collected in each habitat during the collection period of 2013-2014.

Figure 2: The Human-baited Double Net trap method

Vectorial capacity

To understand the importance of different vector species in the transmission of disease often the vectorial capacity is calculated. This vectorial capacity (R0) is the number of malaria/dengue cases derived from one infective case before he/she dies or is cured. If the R0 is lower than one, the disease goes extinct. However if the R0 is larger than one the disease will be able to establish itself in the area.

$$R0_{malaria} = \frac{ma^2bp^n}{-\ln(p)r} \qquad R0_{dengue} = \frac{a}{r}ma^2e^{-\mu n}\frac{bd}{\mu}$$

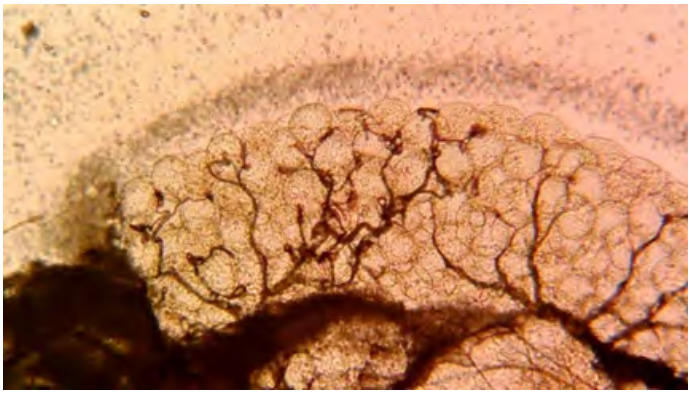
Table 2: Value of formula and description

Value	Description
ma	Vector exposure/per day/per person
a	The frequency of feeding on a person daily rate
b	Proportion of female mosquitoes developing parasites/viruses after taking infective blood meal
p	Probability to survive one day
n	Development days of parasite/viruses in mosquito
r	Rate of human recovery
μ	Mortality rate of female mosquitoes
d	Proportion of people becoming infected after being bitten by an infective mosquito

To calculate this capacity we needed to conduct an additional study in the field to identify the parity rate of malaria and dengue vectors in the field. In June and July 2015 we collect *Anopheles* and *Aedes albopictus* mosquitoes for 42 nights using the human-baited double net traps in the village, secondary forest, immature and mature rubber plantation of village Thinkeo. Mosquito were collected by two participants in each habitat for 6 hours during the highest collection times of malaria vectors according to previous fieldwork in the area, from 17:00 to 23:00.

After identification, the abdominal condition of all female *Anopheles* and *Aedes albopictus* mosquitoes were noted in the laboratory form and grouped as unfed, freshly fed, half-gravid, and gravid. All unfed and freshly fed *Anopheles* and *Aedes albopictus* mosquitoes were dissected and parity was determined using the Detinova method [47]. Females in which the ovaries have coiled tracheolar skeins are nulliparous and have not taken a blood meal yet not have laid eggs (Figure 3A). Ovaries in which the tracheoles have become stretched out are parous and have laid eggs at least once (Figure 3B).

A



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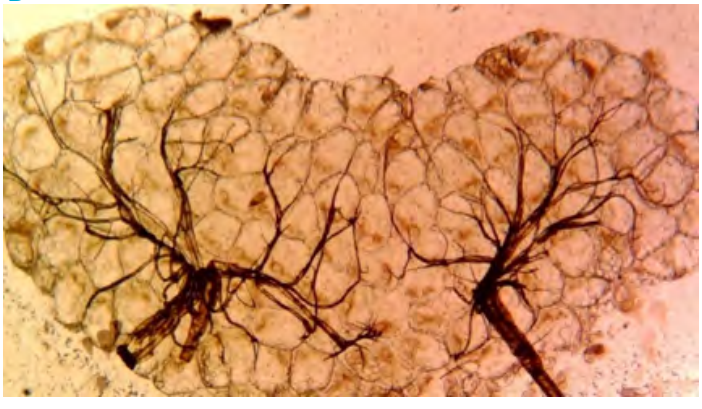


Figure 3: Ovaries dissected from female *Ae. albopictus;* A) nulliparous mosquito with coiled tracheolar skeins B) parous mosquitoes with stretched out tracheolar skeins

Larval survey

To understand where mosquitoes breed in the rubber plantations, a larval survey was conducted from August 2014 until December 2014 on a monthly basis using a systematic approach. Surveys were done in immature rubber plantations, mature rubber plantations and villages. In the mature rubber plantations random collecting cups were checked and all possible breeding sites were analysed. Data on water quality of all identified water bodies, their size, turbidity, location, presence of mosquito larvae and other insects were noted. Mosquito larvae were collected and reared at the field laboratory for identification purpose. Surveys were conducted monthly with the help of two villagers (Figure 4).

Figure 4: Larval survey conducted by our team and local villagers

Molecular data, malaria vector species identification

The *Anopheles* complexes collected during the longitudinal study were analysed for species in Kasetsart University. DNA of *Anopheles* specimens was extracted according to procedures of Linton *et al*. 2001 and Manguin *et al*. 2002 [48, 49]. Molecular identifications were performed using the AS-PCR assay of Garros *et al*. 2004 with primers specific for *An. minimus s.s.* and *An. harrisoni* for the *An. minimus* complex, the AS-PCR assay of Walton *et al*. 2007 with primers specific for *An. maculatus s.s*. and *An. sawadwongporni* for the *An. maculatus* complex and the AS-PCR assay of Walton *et al*. 1999 and Audtho *et al*. 1995 with the specific primers for *An. dirus s.s*., *An. cracens*, *An. scanloni*, *An. baimaii* and *An. nemophilous* for the *An. dirus* complex [50-53].

Molecular data, dengue vector viral identification

No plasmodium detections were conducted on malaria vectors, due to the absence of the disease in our study areas. All collected *Ae. albopictus* samples were screened for arboviral sequences. Additionally all collected *Armigeres kesseli* and *Ar. subalbatus* mosquitoes were screened for arboviral sequences.

The abdomen, wings and legs of samples were separated from the thorax and female and male samples pooled separately in 1-10 samples. RNA was extracted using the NucleoSpin^{R} 8 Virus, Ref: 740 643.5, extraction kit. The RNA of our samples were amplified using specific primers with RT-PCR and screened for the alphavirus and flavivirus genus sequence using agarose gel electrophoresis. The external set of primers Alpha1- KYT CYT CIG TRT GYT TIG TIC CIG G, Alpha1+ GAY GCI TAY YTI GAY ATG GTI GAI GG was used for reverse transcription and external amplification. The internal primers Alpha 2- GCR AAI ARI GCI GCY TYI GGI CC, Alpha 2+ GIA AYT GYA AYG TIA CIC ARA TG were used for the nested PCR [54]. The external set of primers Flavi1- TCC CAI CCI GCI RTR TCR TCI GC, Flavi1+ GAY TYI GGI TGY GGI IGI GGI RGI TGG was used for reverse transcription and external amplification. The internal primers Flavi 2- CCA RTG ITC YKY RTT IAI RAA ICC, Flavi2+ YGY RTI YTY AWC AYS ATG GC were used for the nested PCR [55].

Results January 2015 - December 2015

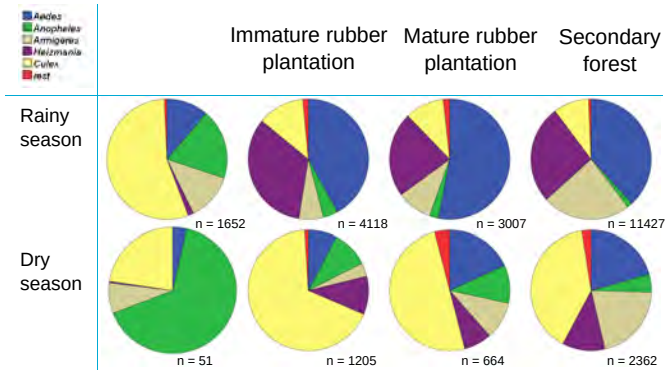
Longitudinal study on mosquito ecology in rubber plantation

Analysis of sampling efficiency was made using generalized estimating equations using a negative binomial model for count data with a log-link function (IBM SPSS statistics, ver. 20). Species diversity was compared using the Simpson's index of diversity (1-D) with the result representing the diversity from 0 (no diversity) to 1 (infinite diversity).

During 3,888 collection hours in each habitat over nine months survey 24,927 female mosquitoes 8.7% were collected in villages (2,164/24,927), 21.4% in immature rubber plantations (5,323/24,927), 14.6% in mature rubber plantations (3,651/24,927) and 55.3% in secondary forest (13,789/24,927) 116 species were collected, including 62 species that have not been recorded in Lao PDR before. 1,249 male mosquitoes were collected of which 71.2% (889/1,249) was *Ae. albopictus*. 13 female and 9 male mosquitoes could not be identified. More than 60% (9,395/15,552) of the sampling occasions yielded no mosquitoes (80%, 3,111/3,888 in village; 59.2%, 2,300/3,888 in immature; 64.7%, 2,514/3,888 in mature; 37.8%, 1,470/3,888 in forest). In the villages 62 mosquito species were collected with a Simpson's index of diversity of 0.864 (95% CI 0.855-0.873). This diversity index was higher than for immature rubber plantations where 79 species were collected (1-D= 0.843 with 95% CI 0.838-848) and mature rubber plantations where 72 species were collected (1-D= 0.816 with 95% CI 0.806-0.825). The secondary forest showed similar species diversity to the villages with 89 species found (1-D= 0.853 with 95% CI 0.850-0.856).

The species distribution was quite similar between the tree habitats (immature rubber plantation, mature rubber plantation and secondary forest) during both the rainy and dry season (Table 3, 4) with a high number of *Aedes* mosquitoes collected in the rainy season and a high number of *Culex* mosquitoes collected in the dry season. However all tree habitats different considerably with the species distribution of the village where the *Culex* mosquitoes were dominant during the rainy season and *Anopheles* mosquitoes during the dry season. In the mature rubber plantations 36.5% (1,331/3,651) of female mosquitoes were *Ae. albopictus*, a vector of dengue. Furthermore 2.5% (93/3651) of female mosquitoes were the malaria vectors *An. minimus s.l.*, *An. maculatus s.l.*, *An. dirus s.l.* and *An. barbirostris.* About 12% (440/3,651) of female mosquitoes were *Cx. vishnui* (vector of Japanese encephalitis).

Table 3: Mosquito species distribution in the village, immature rubber plantation, mature rubber plantation and secondary forest during the rainy season from May to October and the dry season from November to April



Aedes mosquitoes were most abundant, of which 73% (6305/8590) was *Ae. albopictus* mosquitoes. *Aedes* mosquitoes were collected six times more in the rainy seasons (May to October) than in the dry season with rainfall related to higher number of *Aedes* mosquitoes. Compared to the villages exposure to *Aedes* mosquitoes was almost nine times higher in immature rubber plantations ($P<0.0001$), eight times higher in the mature rubber plantations ($P<0.0001$) and almost 25 times higher in the forest ($P<0.0001$) (Table 4).

There was low *Anopheles* activity throughout the study period for all habitats with 50% more collected during the dry season compared to the wet season. *Anopheles* species were host-seeking from 18.00-23.00H with low activity from

0.00-05.00H. In all tree habitats two to three times fewer *Anopheles* mosquitoes were collected than in the villages (all *P*<0.0001) (Table 4).

Armigeres mosquitoes were collected most frequently in the rainy season with two host-seeking behaviour peaks at 6.00-7.00H and 18.00H. Almost 65% (2,621/4,124) of collected *Armigeres* mosquitoes were *Ar. kesseli* mosquitoes. Higher number of *Armigeres* mosquitoes were collected in the tree habitats compared to the villages with 13 times more *Armigeres* mosquitoes collected in the forest (*P*<0.0001) (Table 4).

Most *Heizmannia* mosquitoes were collected during the rainy season with host-seeking behaviour throughout the day. Only few *Heizmannia* mosquitoes were collected in the villages with 50 times more collected in the immature rubber plantations (*P*<0.0001), almost 25 times more in the mature rubber plantations (*P*<0.0001) and 119 times more in the secondary forest (*P*<0.0001) (Table 4).

Culex mosquito collection peaks were present both in the rainy and dry seasons. During the rainy season *Culex* numbers were most abundant in the villages whilst in the tree habitats *Culex* mosquitoes were most abundant in the dry season. Highest host-seeking behaviour was during the evening with cooler temperatures increasing host-seeking behaviour of *Culex* mosquitoes. In the villages 1.6 times more *Culex* mosquitoes were collected than in the mature rubber plantations (*P*<0.0001). However 1.13 times less *Culex* mosquitoes were collected than in the immature rubber plantations (*P*=0.031) and more than two times less than in the secondary forest (*P*<0.0001) (Table 4).

Table 4: Mean catch size of female mosquitoes per person-hour for village compared to immature rubber plantation, mature rubber plantation and secondary forest with odds ratio (OR) and 95% confidence interval (CI) *significantly different, *P*<0.05

	Village		Immature rubber plantation			Mature rubber plantation			Secondary forest		
Mosquito species	**Mean catch/hour (95% CI)**	**OR**	**Mean catch/hour (95% CI)**	**OR (95% CI)**	***P***	**Mean catch/hour (95% CI)**	**OR (95% CI)**	***P***	**Mean catch/hou (95% CI)**	**OR (95% CI)**	***P***
Female mosquitoes	0.56 (0.50- 0.62)	1	1.37 (3.39-3.71)	2.28 (2.12-2.45)	<0.0001*	0.94 (0.88-1.00)	1.56 (1.44 -1.68)	<0.0001*	3.55 (3.39-3.71)	6.77 (6.31-7.26)	<0.0001*
Aedes sp.	0.05 (0.04-0.06)	1	0.47 (0.43-0.51)	8.93 (7.61-10.48)	<0.0001*	0.44 (0.40-0.48)	8.21 (6.99 - 9.65)	<0.0001*	1.25 (1.17-1.32)	25.46 (21.77-29.77)	<0.0001*
Anopheles sp.	0.17 (0.14-0.19)	1	0.07 (0.06-0.09)	0.46 (0.39-0.53)	<0.0001*	0.04 (0.03-0.04)	0.29 (0.19 - 0.28)	<0.0001*	0.07 (0.06-0.08)	0.49 (0.42-0.57)	<0.0001*
Armigeres sp.	0.06 (0.05-0.08)	1	0.08 (0.07-0.09)	1.22 (1.01-1.47)	0.04*	0.09 (0.08-0.10)	1.43 (1.20 - 1.72)	<0.0001*	0.83 (0.75-0.90)	13.16 (11.31-15.31)	<0.0001*
Heizmannia sp.	0.007 (0.00-0.01)	1	0.39 (0.35-0.42)	50.18 (34.46-73.06)	<0.0001*	0.19 (0.17-0.21)	24.53 (16.79 - 35.85)	<0.0001*	0.85 (0.78-0.91)	118.99 (81.86-172.96)	<0.0001*
Culex sp.	0.26 (0.21-0.31)	1	0.34 (0.29-0.40)	1.13 (1.01-1.27)	0.031*	0.17 (0.14-0.19)	0.62 (0.54 - 0.70)	<0.0001*	0.53 (0.46-0.59)	2.09 (1.88-2.33)	<0.0001*

Parity study

During the 42 nights of collection 1,326 vector mosquitoes were collected of which 1,263 were dissected successfully, including 1,171 *Ae. albopictus* mosquitoes and 92 *Anopheles* mosquitoes (82 malaria vectors). Unfortunately number of *Anopheles* malaria vector mosquitoes were very low throughout the collection period with in mature rubber plantations only 10 collected (Table 5). In general the parity rate was high with long living vector mosquitoes present in all habitats.

Table 5: The parity rate of the dengue vector *Ae. albopictus* and malaria vectors in the village, immature rubber plantation, mature rubber plantation and secondary forest

Habitat	Vector species	parity	% parous
Village	*Ae. albopictus*	3/5	60%
	Anopheles malaria vectors	33/35	94%
Immature rubber plantation	*Ae. albopictus*	234/269	87%
	Anopheles malaria vectors	8/23	35%
Mature rubber plantation	*Ae. albopictus*	309/327	92%
	Anopheles malaria vectors	5/10	50%
Secondary forest	*Ae. albopictus*	406/447	91%
	Anopheles malaria vectors	13/14	93%

Vectorial capacity

The vectorial capacity for malaria disease was calculated for *Plasmodium falciparum* and *Plasmodium vivax* parasites separately for each habitat for the three most common and important primary vector collected in our study areas; *An. maculatus s.l., An. minimus s.l.* and *An. dirus s.l.* All habitats show high vectorial capacity during the dry and wet season. It seems that *An. minimus* is the main vector for malaria transmission in the different habitats during the dry season, which shifts to *An. maculatus* during the wet season. *An. dirus* is not an important malaria vector in our study sites. Similar vectorial capacity can be found for both parasite species, with a slightly higher R0 for *P. vivax*. The vectorial capacity of dengue was calculated using the primary vector *Ae. albopictus*. The vectorial capacity in the secondary forest was highest, followed by the mature rubber plantations.

In the next few months the vectorial capacities will be related to the human behavior of the rubber plantation workers and the villagers to understand the risk of infection in each habitat.

Table 6: The vectorial capacity of *P. falciparum* in the forest, mature rubber plantation, immature rubber plantation and village habitats was calculated for dry and wet season using the vectors *An. maculatus s.l.*, *An. minimus s.l.* and *An dirus s.l.*

	Dry season				Wet season			
P. falciparum	**Forest**	**Mature rubber**	**Immature rubber**	**Village**	**Forest**	**Mature rubber**	**Immature rubber**	**Village**
An. maculatus s.l.	13.1	22.1	39.2	11.4	28.6	16.6	64.0	28.6
An. minimus s.l.	18.1	36.1	41.6	84.9	8.3	2.8	6.9	42.8
An. dirus s.l.	0.03	0.2	0.5	0.02	0.2	0.1	0.5	0

Table 7: The vectorial capacity of *P. vivax* in the forest, mature rubber plantation, immature rubber plantation and village habitats was calculated for dry and wet season using the vectors *An. maculatus s.l.*, *An. minimus s.*l. and *An dirus s.l.*

	Dry season				Wet season			
P. vivax	**Forest**	**Mature rubber**	**Immature rubber**	**Village**	**Forest**	**Mature rubber**	**Immature rubber**	**Village**
An. maculatus s.l.	14.9	25.2	44.8	13.1	31.2	18.1	69.8	31.2
An. minimus s.l.	19.3	38.5	44.3	90.6	8.8	2.9	7.4	45.7
A. dirus s.l.	0.05	0.3	1.0	0.05	0.3	0.2	0.7	0

Table 8: The vectorial capacity of dengue in the forest, mature rubber plantation, immature rubber plantation and village habitats was calculated for dry and wet season with the primary vector *Ae. albopictus*

Dry season				Wet season			
Forest	**Mature rubber**	**Immature rubber**	**Village**	**Forest**	**Mature rubber**	**Immature rubber**	**Village**
10.6	2.8	1.5	0.01	42.0	18.8	9.5	0.06

Larval survey

During the five monthly surveys in immature rubber plantations, mature rubber plantations and villages 1,384 waterbodies were surveyed. A total of 52% (725/1,384) of waterbodies surveys were positive for mosquito larvae. Interestingly we found *Culex*, *Armigeres* and *Aedes* mosquito larvae in the latex collection cups, even when latex was still present. *Aedes* larvae were furthermore found breeding in latex collection cups, banana plant axils, leaves of banana plants and in garbage discarded by rubber workers, including tires and coconut shells. We found *Anopheles* mosquitoes breeding in road side puddles and in small puddles next to the streams (Figure 5).

Figure 5: Larval breeding sites, including old discarded tires, leaves of banana plants and latex collection cups

Molecular data, malaria vector species identification

Unfortunately none of the subspecies of *An. dirus* could be identified due to problems with the PCR at Kasetsart University. However for *An. minimus* and *An. maculatus* the following species were identified.

An. minimus complex

- *Anopheles minimus* 86 samples
- *Anopheles aconitus* 57 samples
- *Anopheles varuna* 1 sample

An. maculatus complex

- *Anopheles maculatus* 180 samples
- *Anopheles pseudowillmori* 35 samples
- *Anopheles sawadwongporni* 9 samples
- *Anopheles dravidicas* 10 samples

Molecular data, dengue vector viral identification

We tried to identify the vector of dengue in our study area where during our study period from July 2013-June 2014, 199 dengue cases were reported in study district Xieng Ngeun and 11 cases in Nane district. All samples of *Aedes albopictus* were screened for the presence of the alphavirus and flavivirus in pools of 1 to 10 mosquitoes. A total of 1,252 *Ae. albopictus* pools were tested for alphavirus and flavivirus sequences. None of the pools displayed amplicon of expected size for alphavirus. For flavivirus, 36 positive pools were identified. The positive pools of flavivirus were from both male and female mosquito pools and mosquitoes collected from mature rubber plantations, immature rubber plantations and secondary forest. Further analysis of these positive pools has not given any information on the specific flavivirus present in the samples.

The possible secondary dengue vector *Armigeres kesseli* / *Ar. subalbatus* was also analysed for alpha and flavivirus presence. Of the 510 pools tested, none displayed amplicons of expected size.

National communication

The emphasis of this ECOMORE project is on the communication with the stakeholders within Lao PDR. We have therefore organized multiple village meetings in our study areas throughout 2015, with our final one conducted in September 2015. These village meetings were organized to share our results and recommendations and have close relations with the villages we work with. Furthermore an ECOMORE National Stakeholders meeting was organized in Vientiane Capital to discuss our findings and recommendations with the government and other important stakeholders within the country. Moreover we participated in the Strategic Plan for Health meeting in Luang Prabang province and shared our results with the Department of Communicable Disease Control, Ministry of Health of Lao PDR (Figure 6).

Figure 6: Images of our village meeting, National Stakeholders meeting and participation in the Strategic Plan for Health meeting

Currently we are also in the process of including a health Chapter in the government Rubber Information book (both Lao and English language). This chapter focussed on work-related injuries (risks and prevention), environment-related injuries (risks and prevention) and pathogenic diseases (risks and prevention indoor, outdoor and larval source management). The inclusion of this chapter in the information book will hopefully make rubber industry stakeholders more aware of the risks related to working in rubber plantations and how to prevent these.

Interational communication

Apart from communication of our project and results within Lao PDR we attended several meetings to share our results. We gave an oral presentation at the 3rd GRF One Health Summit 2015 in Davos, Switzerland. Furthermore we presented a poster at the International Scientific Symposium - Institut Pasteur International Network 2015 in Paris, France. We also presented our work at the Vientiane International symposium on ethics in research for international development (Figure 7). Additionally we published our first scientific paper in the open source journal of PloS One, named 'The Human-Baited Double Net Trap: an Alternative to Human Landing Catches for Collecting Outdoor Biting Mosquitoes in Lao PDR'.

Figure 7: Images of the GRF One health summit presentation, the poster presentation at the International Scientific Symposium of Pasteur Paris and the presentation at the Vientiane International symposium on ethics

Training provision

The ECOMORE project not only conducted research, we also trained an entomologist from Myanmar, Mr. Saw Mitchell, at our institute in Lao PDR for 6 weeks. He received basic training in entomology, including field training to collect mosquito larvae, adult mosquito identification, training in different collection techniques (CDC light trap, BG sentinel, Human-baited Double Net trap, Human Landing Catches) and how to work in the entomological colony room (Figure 8).

Figure 8: Images of the training of Mr. Saw Mitchell; the team, the fieldwork and the laboratory work

Opportunities in Africa

The ECOMORE project has been such a success that we have now started working together with Institut Pasteur de Côte d'Ivoire to do a mirror study in their country where malaria is endemic. This south-south cooperation between the two institutes will be a great way for two developing countries to share knowledge and experience. Especially for the Lao team to share their knowledge and experience from the ECOMORE project will be invaluable for the success of the Ivorian project. The Lao team has visited the project in Côte d'Ivoire as has the team from Côte d'Ivoire visited the field sites and laboratory in Lao PDR (Figure 9).

Figure 9: Images of the two teams working together; in the field and in the laboratory

Future activities

The ECOMORE project will come to an end in July 2016. Before the end of the project we will focus on finishing the rubber information book. We will work closely together with NAFRI to ensure the updated book, including our health chapter, will be available for the rubber industry stakeholders before the end of our project. We will finish the analysis of the longitudinal study and larval survey and publish our results in scientific journals. Most importantly we will conduct a study in the field to compare different commercially available methods in which rubber workers can protect themselves from mosquito bites whiles tapping latex. The results from this study will be communicated to the interested stakeholders to ensure a proper recommendation can be made concerning protection methods in the plantations.

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Risk of Vector-borne Diseases in Relation to Rubber Plantations in Côte d'Ivoire as compared to Lao PDR

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Funded by: The Michelin Foundation

Background

In March 2013 we started the ECOMORE project on risk of vector-borne diseases in relation to rubber plantations in Lao PDR. This study is of importance in Lao PDR where an estimated 300,000 people will work in rubber plantations in the near future. Mosquitoes were collected using a double bed net method in Luang Prabang province in four different habitats; secondary forest, immature plantation, mature plantation (yielding latex) and village. A total of 19,466 mosquitoes were collected and identified in 5 months in the four different habitats, consisting of 113 different species. Of these mosquitoes 1,641 possible dengue and chikungunya vectors and 250 possible malaria vectors were collected in the rubber plantation. We therefore identified putative malaria and dengue vectors are present in the rubber plantations. This indicates the possibility of vector-borne disease transmission occurring in the plantations and showed us the times during the day and during the year when risks of exposure were highest for the workers and their families. Using this information we can advise a proper vector control method for the rubber workers to decrease their risk of vector-borne diseases.

While doing this study we realized the data we were collecting is not only interesting for Asia, but could also be of importance in other continents where a lot of rubber plantations are present and many vector-borne diseases occur. It is known that malaria is actively transmitted in and around rubber plantations in Côte d'Ivoire and it is of vital importance to understand the behavior of these mosquitoes in the rubber plantation area to advice a proper control method. Not only that, comparing the data of this mirror study with data from Lao PDR will give us a more comprehensive knowledge of the mosquito ecology and their behavior in rubber plantations. This generalization can help protect staff working in rubber plantations all over the world.

Rationale

Areas of West Africa, such as Côte d'Ivoire, have extensive natural rubber production. We anticipate that the changes in the environment to rubber cultivation will result in an altered risk from vector-borne diseases for the workers, their families and surrounding villages; predominantly malaria and yellow fever, but also arboviral diseases such as dengue and chikungunya. This study will provide an opportunity to further develop the entomological department at Institut Pasteur de Côte d'Ivoire whilst deepening our understanding of the vector ecology in rubber plantations in West Africa. It is envisaged that this project will allow us to advise different governmental and health organizations how to decrease vector-borne disease incidence in rubber plantation areas where active transmission of vector-borne diseases already occur. Furthermore this data will be combined with data collected in a similar way in Lao PDR to write a comprehensive document about the vector-borne disease risks for rubber workers. This study will be of relevance to public health workers, governments and those working in the rubber industries of Côte d'Ivoire, other countries in West Africa and South-East Asia.

Overall goal: To assess the potential risk of vector-borne disease infections occurring in and around rubber plantations

Objectives:

- Determine the risk of mosquito-borne diseases like malaria, yellow fever, dengue and chikungunya arising in rubber plantations compared to surrounding villages
- Understand when and where rubber plantation workers are exposed to the vector mosquitoes
- Provide advice on how to decrease exposure of rubber plantation workers and their families to vector-borne diseases

- Understand the differences and similarities between Laos and Côte d'Ivoire in vector-borne disease risk for rubber plantation

Methods

The project officially started in 2015 and is currently in the start-up phase. No data has been collected yet, with work still in the preparatory phase.

Field sites selection

During two visits to Côte d'Ivoire by the Lao team (March 2014 and February 2015), the Ivorian and Lao team tried to identify suitable field sites by visiting together several rubber plantation areas close to Abidjan city. We tried to find three villages surrounded by mature and immature rubber plantation and one large village without any rubber plantation in the area. After visiting several sites, four study areas with three habitats were selected (table 1). The three study areas Agnimangbo, Adangba-Eby and Kotokodji consist of a small village surrounded by rubber plantations. In these study areas entomological surveys will be conducted in the village, in the surrounding immature rubber plantation (i.e. not tapped for latex) and in the surrounding mature rubber plantation (i.e. tapped for latex for at least 10 years). In Lopou, a large village without rubber plantations, entomological surveys will be conducted in three sites. These three sites within Lopou village will serve as the control to understand the difference in risk of mosquito-borne diseases.

Table 1: Study sites of the Yersin project: the four study areas with three habitats

Study area	Habitat
Agnimangbo	Village surrounded by rubber plantations
	Immature rubber plantation
	Mature rubber plantation
Adangba-Eby	Village surrounded by rubber plantations
	Immature rubber plantation
	Mature rubber plantation
Kotokodji	Village surrounded by rubber plantations
	Immature rubber plantation
	Mature rubber plantation
Lopou	Village site not surrounded by rubber plantations
	Village site not surrounded by rubber plantations
	Village site not surrounded by rubber plantations

Rapid rural appraisals

Before the start of the entomological survey a rapid rural appraisal will be conducted in all four villages to understand the behavior and attitude of the villagers towards vector-borne diseases. This method enables our team members to introduce our project and involve villagers, by asking them to share their opinions and ideas on vector-borne diseases in an interactive way. These appraisals will also be used to get a village layout and an overview of the areas in the village with high number of mosquitoes.

Human-baited double net collections

Adult mosquitoes will be collected in the three habitats of our four study areas using the human-baited double net collection method. Participants are positioned on a bed, protected from mosquito bites using a bed net. A second bigger bed net is hung over the small bed net with a gap at the bottom (Figure 1). Through this gap between the two bed nets mosquitoes attracted to the participant enter and are caught. Every hour the participant inside the small bed net collects the mosquitoes in between the bed nets for 10 minutes. Mosquitoes collected with the human-baited double net method are frozen and identified to species.

Figure 1: Schematic overview of the human baited double net trap

Parity status of vector mosquitoes

To understand the longevity of the vector mosquitoes and therefore their vectorial capacity, the vector mosquitoes collected with the human-baited double net method will be analyzed for parity. The ovaries of the important dengue, yellow fever and malaria vectors are dissected and identified to parous or nulliparous ovaries. Every season (rainy or dry) at least 100 mosquitoes of each vector species will be dissected. This includes, if present, the mosquito species *Aedes albopictus*, *Aedes aegypti*, *Anopheles gambiae s.l.* and *Anopheles funestus.*

Quick seasonal survey

Our entomological survey needs to be related to human behavior for analysis of vector-borne disease risk. We will

therefore collect the human behavior data using quick seasonal surveys in all four villages. Questions will be asked about their mosquito protection behavior and their activities the day before the survey in a seven minute long questionnaire. The survey will be conducted on a predetermined number of randomly chosen villagers and rubber workers (to be determined later using household data) every season. This seasonal behavioral data of villagers and rubber workers will be related to the seasonal entomological data.

Health and environment data

Data on disease prevalence, environmental description and weather needs to be collected from the health and agricultural department of the Ivorian government. As there is huge amount of data available, a new SOP is currently being written to clearly define which data we would need and how we could receive this data from departments within the government. Furthermore, in the SOP we detail how this data would be integrated in our analysis and results.

Communication

We will provide a three-monthly newsletter with a general update of the project, a road map with our accomplishments so far and a scientific update. In January 2016, the Michelin Corporate Social Responsibility team will visit the project in Côte d'Ivoire both in the field sites and in the entomology laboratory in Abidjan.

Future activities

1. Receive equipment for the field laboratory
2. Conduct Rapid Rural Appraisals in all four study villages
3. Start the human-baited double net collections in the four villages with a total of 12 collection sites
4. Conduct parity study on vector mosquitoes collected
5. Conduct the quick seasonal survey in the four villages

Vector mapping, characterization of insecticide resistance of Aedes populations, and entomology capacity development in Lao PDR

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Noy Khounsombat, (Laboratory technician)
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Bounma Xayasouk (Mosquito insectary technician)

Background

Because of global changes (environment, climate) and increasing transportation, in the past decades we have seen a dramatic resurgence of dengue and chikungunya throughout regions where *Aedes* mosquito vectors are present and this has led to major public health problems (WHO 2006, Benedict *et al*., 2007). In 2013, Lao PDR faced one its most severe dengue outbreak in years (Figure 1). The mosquito *Aedes aegypti* is the main dengue virus vector in Lao PDR (Dr Vongphayloth pers com.). Another species, *Aedes albopictus*, is a secondary vector of dengue, but is also the vector of the chikungunya virus that is reemerging in Southeast Asia and in Lao PDR. Because there is still no vaccine or specific treatment available against these viruses, vector control remains the only strategy for reducing dengue or chikungunya transmission. Effective vector control measures rely on active community participation, health education programs, and environmental management that include improvement of water supplies and storage, solid waste management, and modification of human-made larval habitats (Erlanger *et al*., 2008). During inter-epidemic periods or when the elimination of breeding habitats of the mosquito is not easily achievable, insecticide application in larval habitats is routinely conducted by public health services in many countries including Lao PDR (Thavara *et al*., 2004). Space spraying applications are conducted during epidemics or when the entomological indices of mosquitoes are high. For both larviciding and adulticiding, organophosphates and pyrethroids are the insecticide families of choice worldwide as well as in Lao PDR.

Unfortunately, many dengue vector control programs are threatened by the development of insecticide resistance in *Aedes* populations across the world. Insecticide resistance is associated with mutations in the sequence of the target protein that induce insensitivity to the insecticide (target-site resistance, knock-down resistance mutation, *kdr*), and/or the up-regulation of detoxification enzymes (metabolic-based resistance, P450 monooxygenases (P450s), glutathione S-transferases (GSTs) and carboxy/cholinesterases (CCEs)).

Strong levels of resistance to organophosphates and pyrethroids have been detected in *Aedes aegypti* population in Southeast Asia (Ranson, 2010). Resistance to these same families of insecticide has also been detected in *Aedes albopictus* populations worldwide (Kamgang *et al* 2010, Marcombe *et al.* 2014). The organophosphate temephos (larvicide) and insecticides from the pyrethroid family (permethrin, deltamethrin; adulticides) have been used in Lao PDR for decades to reduce the vector populations during important dengue epidemics but to date, compared to its neighboring countries there is no information available on the resistance status of *Aedes* populations and the possible impact of the resistance on vector control operations in the country.

The risk of insecticide resistance in dengue vectors in South-East Asia represents a serious threat to the achievements seen in dengue control during recent years. It is urgent to identify the distribution, the levels, the mechanisms, and potential environmental factors of resistance in dengue vectors in the lower Mekong countries to assist health authorities to develop more effective strategies of prevention and control of the disease.

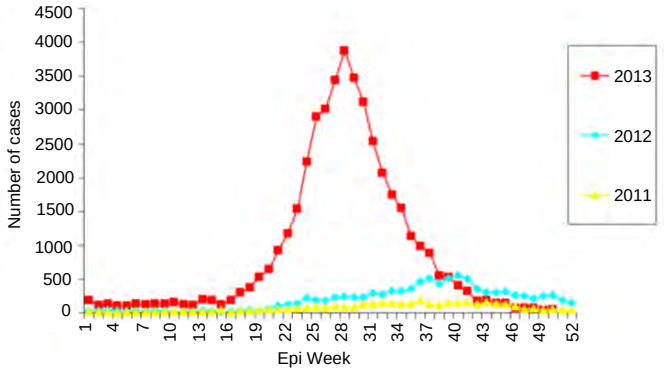

Figure 1. Dengue cases in Lao PDR, 2011 – 2013. Data from Source: http://www.wpro.who.int/emerging_diseases/documents/dengue.updates.2013/en/

Objectives and expected outcomes.

The purpose of this project is to provide entomology capacity building, identify circulating levels of insecticide resistance (IR), and improve data on vector risk profiles in high priority location in Lao PDR.

Objectives:

- Evaluation of the levels of insecticide resistance in the vector populations in Lao PDR.
- Evaluation of the types and mechanisms of insecticide resistance (i.e. metabolic or target site) in Laos.
- Evaluation in semi-field trials of common insecticide formulations used in Lao PDR versus candidate insecticides for larval control
- Capacity building in medical entomology in Lao PDR.

Expected outcomes:

- Set up a comprehensive map of the levels of insecticide resistance in Lao PDR
- Generate an Insecticide Resistance database in the main dengue vectors in Lao PDR
- Guide public health authorities of Laos in the design and implementation of Insecticide Resistant Management strategies.
- Capacity strengthening in medical entomology and vector control in Lao PDR.

Methods and Results

Mosquito collections:

During the rainy season of 2014, collections were made in Vientiane capital, Xayabury and Luang Prabang provinces and larvae collected by collaborators from Saravane and Attapeu (CMPE and district staff) were sent to the Institut Pasteur. In Xayabury province, larvae were collected in breeding habitats in several villages and brought back to the laboratory for rearing. Larvae were collected in the forest surrounding Tinkheo village and in rubber plantations (rural areas) in Luang Prabang province. Larvae were also collected in Luang Prabang city in households and in temples (urban area). Sentinel sites were established in Vientiane Capital in several districts and villages and larval collections were made every week at these locations. All the collection sites were geo-referenced (Figure 2 & Table 1). Another collection was made in Luang Prabang in July 2015 to collect *Ae. albopictus* populations. (Figure 3).

Figure 2. Location of the mosquito collection sites.

Table 1. List of the *Aedes aegypti* populations collected and their GPS coordinates.

Province	District	Village	GPS coordinates	
Xayabury	Borten	Taling	17.784729°N	101.170521°E
Luang Prabang	Luang Prabang	Khomkhuang	19.902775°N	102.156213°E
		Thatnoy	19.531432°N	102.075364°E
		Thongchaleun	19.887366°N	102.132352°E
Vientiane Capital	Saythany	Oudomphon	18.125733°N	102.665011°E
		Phailom	18.057037°N	102.774993°E
	Chanthabouly	Dongpalab	17.988083°N	102.605268°E
	Sisattanak	Kao-ngot*	17.962684°N	102.615035°E
Saravane	Lakhonepheng	Lakhonepheng	18.33284°N	103.05372°E
	Vapi	Khonsaiy	15.53837°N	103.34010°E
Attpeu	Samakheexay	Xaysa-art	14.8114°N	106.83722°E

*IPL strain, collected at the Institut Pasteur du Laos

Figure 3. Larval collection of dengue vectors in Luang Prabang province, July 2015.

Morphological mosquito identification

For all the mosquito populations collected, larvae were reared until adults (F1 generation; Figure 4). After adult identification, mosquitoes obtained were separated by species and location. Only *Aedes aegypti* and *Ae. albopictus* were kept for breeding. Females mosquitoes were then blood fed using quail and the eggs obtained were kept for the larval and adult bioassays.

Figure 4. Colony room at the Institut Pasteur du Laos.

Insecticide resistance status

We tested the susceptibility of *Aedes aegypti* mosquitoes to a range of insecticides representative of those historically and currently used for mosquito control in Lao PDR (ie DDT, temephos, malathion, deltamethrin and permethrin). Larval and adult bioassays were be performed following WHO guidelines (WHO 2005, 2006; Figure 5).

Larval bioassays on *Ae. aegypti* were performed using late third- and early fourth-instar larvae of the field strains. For each bioassay, larvae of each strain were transferred to cups containing 99 mL of distilled water and 1 mL of the insecticide tested at the desired concentration. Five cups per concentration (25 larvae per cup) and 5–8 concentrations in the activity range of each insecticide were diluted in ethanol. USDA (reference susceptible strain) and field strains were considered as having different susceptibility to a given pesticide when the ratio between their $LC_{50/95}$ or $LD_{50/95}$ (resistance ratio: $RR_{50/95}$) had confidence limits excluding the value of 1. A mosquito strain is considered susceptible when its value of RR_{50} is less than 5, moderately resistant when RR_{50} is between 5 and 10, and highly resistant when RR_{50} is over 10. Larval bioassays on the *Ae. albopictus* populations were run with diagnostic doses (WHO recommended) of deltamethrin (0.00132 mg/L), permethrin (0.014 mg/L), temephos (0.02 mg/L) and DDT (0.04 mg/L). Mortality was recorded after 24h. Following WHO criteria, a population is considered resistant if the mortality after 24 h is under 90%, resistance is suspected with mortality between 90 and 98% and a population is susceptible with mortality over 98%.

Adult bioassays were run using filter papers treated with diagnostic doses of deltamethrin (0.05%), permethrin (0.25%), DDT (4%), and malathion (0.8%). Mortality resulting from tarsal contact with treated filter papers was measured using WHO test kits on adult mosquitoes of the different populations. Four batches of 25 non-blood-fed females (2–5 days of age) were introduced into holding tubes and maintained for 60 minutes at 27 ± 2°C and a relative humidity of 80 ± 10%. Insects were then transferred into the exposure tubes and placed vertically for 60 minutes under subdued light. Mortality was recorded 24 hours after exposure.

Figure 5. Larval and Adult bioassay.

Results of the larval bioassays on the *Ae. aegypti* populations are shown in Tables 2. For each strain and each insecticide, the dose mortality relationships were fitted by regression (P>0.05). For each bioassay, when control mortality was greater than 5% but less than 20%, then the observed mortalities were corrected using Abbott's formula. The resistant ratios (RR) were calculated using the susceptible reference strain (USDA).

The *Aedes aegypti* populations from Taling (Xayabury province), Lakhonepheng (Saravane province), and Oudomphon village showed the highest resistant ratios to temephos (RR_{50}>3). The other populations had significant RR_{50} and RR_{95} but like the previously cited cannot be considered as fully resistant. All the populations showed a tolerance to the insecticide deltamethrin, with significant RR_{50} (>6.25) and RR_{95} (>2.3). The populations from Saravane and Vientiane Capital (Phailom village) showed the highest resistance levels with RR_{50} of 17.5 and RR_{95} of 10.3, and RR_{50} of 18.8 and RR_{95} of 5.9, respectively. All the population tested against permethrin exhibited low to high resistance to the insecticide with RR_{50} between 2 and 15.2 and RR_{95} between 3.6 and 22.8. *Aedes* mosquitoes from Vientiane were the most resistant to permethrin. (RR_{50}>13 and RR_{95}>22). All the population tested against DDT exhibited high resistance to the insecticide with RR_{50} between 7.6 and 171.7 and RR_{95} between 6.9 and 89.3. *Aedes* mosquitoes from Taling in Xayabury province were the most resistant to DDT (RR_{50} = 171.7 and RR_{95} = 89.3).

Table 2: Resistance status of *Aedes aegypti* populations (larvae) against temephos, deltamethrin, permethrin and DDT.

		temephos		deltamethrin		permethrin		DDT	
Province	Strain	RR_{50}*	RR_{95}*	RR_{50}*	RR_{95}*	RR_{50}*	RR_{95}*	RR_{50}*	RR_{95}*
Vientiane Captial	Kao-ngot	1.6	1.9	13	8	15	23	96	60
	Dongpalab	2.2	2.9	8	4	-	-	100	81
	Phailom	1.9	1.8	19	6	-	-	124	75
	Oudomphon	3.5	2.6	14	3	14	24	95	46
Luang prabang	Khomkhuang	1.6	1.7	7	3	-	-	24	18
	Thatnoy	1.9	1.8	12	4	-	-	83	44
	Thongchaleun	2.3	2.1	12	6	-	-	19	70
Saravane	Khamsaiy	2.4	4.2	6	2	-	-	10	7
	Lakhonpeng	3.3	6.5	18	10	10	14	72	48
Attapeu	Xaysa-art	1.8	2.2	6	2	2	4	8	9
Xayabury	Taling	3.6	3.6	8	3	9	16	172	89

LC = Lethal concentration, *Resistant ratios = LC_{50} of wild strain / LC_{50} of USDA susceptible reference strain.

Results of the larval bioassays on the F1 *Aedes albopictus* populations are shown in Table 3. The result showed that all the populations tested were resistant to DDT, except in Oudomphon were resistance is suspected. All the populations were susceptible to permethrin. In Luang Prabang province, resistance to temephos is suspected. In Vientiane province, both population tested were resistant to temephos and the population from Suanmone was resistant to permethrin.

Table 3: Resistance status of *Aedes albopictus* (larvae) against DDT, temephos, malathion deltamethrin and permethrin

Province	Village	Insecticide	n	% Mortality	Status
Luang Prabang	Huayhoy	DDT	145	17	Resistant
		Temephos	100	74	Resistant
		Malathion	200	100	Susceptible
		Deltamethrin	150	900	Resistant
		Permethrin	150	100	Susceptible
Luang Prabang	Phomphao	DDT	150	11	Resistant
		Temephos	200	92	Resistance supected
		Malathion	200	100	Susceptible
		Deltamethrin	150	91	Resistance supected
		Permethrin	200	0	Susceptible
Vientiane	Suanmone	DDT	150	28	Resistant
		Temephos	125	42	Resistant
		Malathion	200	100	Susceptible
		Deltamethrin	100	82	Resistant
		Phermetrin	150	100	Susceptible
Vientiane	Oudomphon	DDT	50	98	Resistance supected
		Temephos	200	87	Resistant
		Malathion	200	100	Susceptible
		Deltamethrin	50	94	Reistance supected
		Phermetrin	50	100	Susceptible

The diagnostic dosages of DDT, temephos, malathion, deltamethrin and permethrin used were 0.04 mg/L, 0.02 mg/L, 1 mg/L, 0.00132 mg/L and 0.014 mg/L, respectively.

The results of the adult bioassays are shown in Figure 6. *Aedes aegypti* populations from Vientiane, Luang Prabang, Saravane, Attapeu and Xayabury were tested. For each bioassay, when control mortality was greater than 5% but less than 20%, the observed mortality was corrected using Abbott's formula. As expected, the susceptible reference strain (USDA) showed full susceptibility to the four insecticides tested. All the populations tested were highly resistant to the organochlorine DDT with resistance levels varying from zero to thirty-one percent mortality. The most resistant population was in Thongchaleun village in Luang Prabang province (0% mortality). All the populations tested against permethrin (pyrethroid family) presented high levels of resistance with mortality rates values between seven and eighty-three percent.

The most resistant population was in Thongchaleun village in Luang Prabang province (0% mortality). All the populations tested against permethrin (pyrethroid family) presented high levels of resistance with mortality rates values between seven and eighty-three percent. Most of the mosquito populations were susceptible to deltamethrin, which is also an insecticide from the pyrethroid family. Only one population, from Vientiane province (Dongpalab village), showed a moderate resistance to this insecticide (87% mortality). All the populations tested against malathion, an insecticide from the organophosphate family, were resistant to this insecticide (mortality<80%) or showed the beginnings of a resistance (mortality between 91% and 99%).

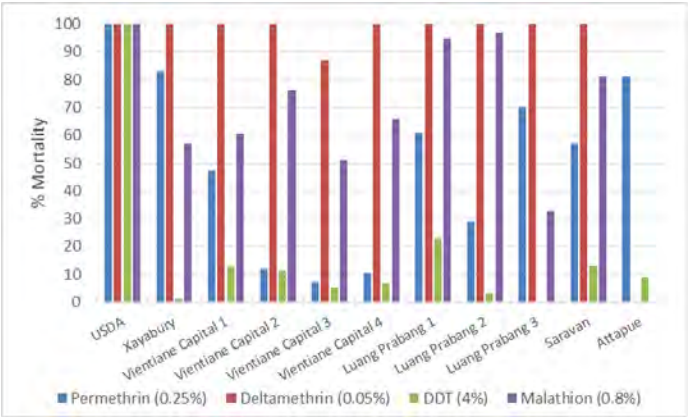

Figure 6. Mortality rates in WHO tube tests of *Aedes aegypti* from Lao PDR.
USDA: susceptible reference strain; Vientiane Capital 1: Kao-Gnot; V. Cap. 2: Phailom; V. Cap. 3: Dongpalab; V. Cap. 4: Oudomphon; Luang Prabang 1: Khomkhuang; LPB 2: Thatnoy; LPB 3: Thongchaleun.

Insecticide resistance mechanisms

Detoxification enzyme activities

The levels P450s, and the activities of CCEs and GSTs were assayed from single 3 day-old F1 females following the microplate methods described by Hemingway (WHO, 1998) and Brogdon (1997) on a spectrophotometer. Total protein quantification of mosquito homogenates were performed using Bradford reagent with bovine serum albumin as the standard protein in order to normalize enzyme activity levels by protein content. For P450s assays, the OD values were measured at 620 nm after 30 min incubation of individual mosquito homogenate. Nonspecific α- and β-CCEs activities were assayed by 10 min incubation of mosquito homogenate in each well with 100 µL of 3 mM napthyl acetate (either α- or β-) at room temperature and the OD values were measured at 540 nm. The activities were determined from α- or β-naphtol standard curves. Glutathione-S-transferases (GST) activity were measured in kinetic at 340 nm for 20 min, and the activity was expressed in nmoles GSH conjugated/min/mg protein.

All the populations tested showed significant higher quantity of P450s compared to the susceptible reference strain. Most of the populations except the population from Luang Prabang showed higher GSTs activities compared to the susceptible reference strain USDA. All the populations tested presented higher alpha and beta-Esterases activities than the susceptible strain (Figure 7).

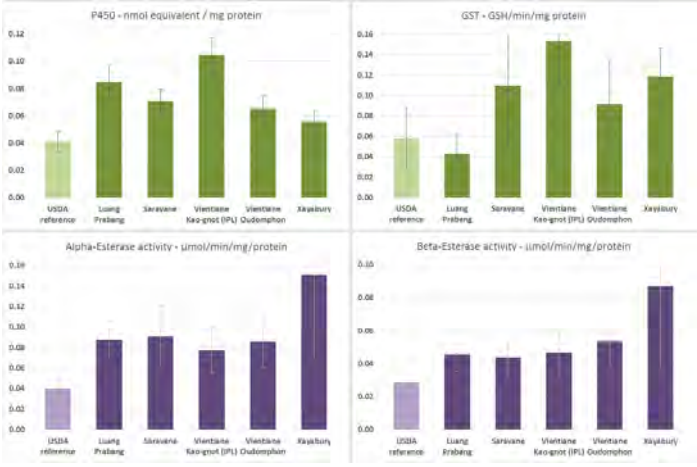

Figure 7. Global amount or activity of detoxification enzymes in *Aedes aegypti* larvae from field populations and the reference strain (USDA). Cytochrome P450 monooxygenases (P450s), Esterase (α and β-CCEs), and Glutathione-S transferases (GSTs). Sample sizes are 47 specimens/population). Confidence intervals are one standard deviation of the mean

KDR detection

Pyrethroid resistance can be due to metabolic detoxification and/or target site modification through non silent points of mutations (*kdr*) in the gene encoding for the Voltage-Gates Sodium Channel (Hemingway *et al*., 2004). In *Aedes aegypti* the known kdr mutations conferring pyrethroid resistance are located at loci 1016 (Val→Gly) and 1534 (Phe→Cys) (Brengue *et al.*, 2003; Harris *et al*., 2010). Both mutations are present in *Ae. aegypti* in South-East Asia (Yanola *et al.*, 2011; Kawada *et al*., 2009 and 2014) and are considered as relevant molecular markers to investigate pyrethroid resistance (Figure 8).

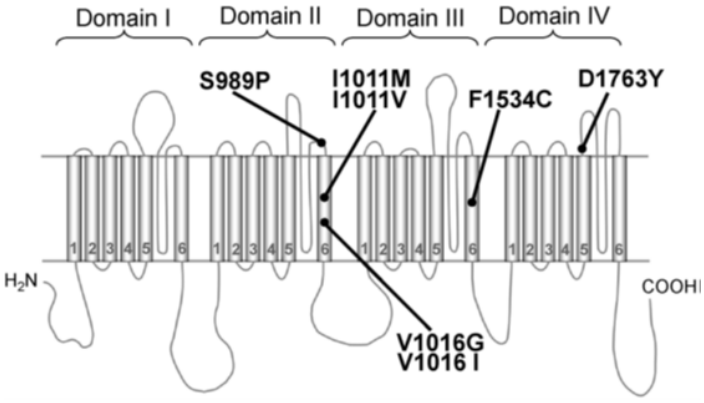

Figure 8. Diagram of the locations of possible kdr mutations found in *Aedes aegypti*. Point mutations in the voltage-gated sodium channel protein so far reported from pyrethroid-resistant *Ae. aegypti* are indicated.

Molecular detection of the V1016G substitution

The detection of V1016G mutation in the voltage-sensitive sodium channel has been performed by qPCR based on the High Resolution Melting (HRM) curve developed by Saaverda-Rodriguez *et a*l. in 2007. A total of 1,076 females of *Aedes aegypti* coming from 11 field-caught populations were tested by real-time PCR to detect the presence of the V1016G and F1534C kdr mutations.
The genotypic distribution and kdr frequency for the V1016G kdr mutation are shown in Table 4. The 1016G mutation was found at low frequency and range from 0 (Khonsaiy & Xaysa-art) to 0.36 (Dongpalab). Significant differences in the frequency of the 1016G allele were found between populations (pair-wise comparisons, P<0.05). All populations were at hardy Weinberg Equilibrium except Khomkhuang (P=0.0043) and Thongchaleun (P<0.001).

Table 4. Genotype distribution and allelic frequency of the V1016G kdr mutation

Province	Strain	SS	RS	RR	F(R)	Fis	HW(p value)*
Vientiane Capital	Kao-gnot IPL	86	30	2	0.14	-0.0266	1
	Dongpalab	34	40	10	0.36	-0.0311	0.82
	Oudomphon	53	43	12	0.31	0.0742	0.504
	Phailom	90	8	0	0.04	-0.0374	1
Luang Prabang	Taling	91	24	1	0.11	-0.0353	1
	Khomkhuang	102	4	2	0.04	0.4843	0.004
	Thongchaleun	81	6	18	0.2	0.823	0
Saravane	Thatnoy	87	28	2	0.14	-0.0093	1
	Lakhonepheng	101	13	1	0.07	0.0772	0.386
Attapeu	Khonsaiy	50	0	0	0	N/A	N/A
Xayabury	Xaysa-art	50	0	0	0	NA	N/A
	Total	**825**	**196**	**48**			

**Hardy Weinberg equilibrium tested by exact-test Fisher. In bold, significant (p-value<0.05) population that showed difference from HW expectations.*

Molecular detection of the F1534C substitution

The detection of the F1534C mutation on the voltage-sensitive sodium channel was based on the HRM method developed by Yanola and colleagues and adapted. by our team. The principle is the same as for the detection of the V1016G mutation The genotypic distribution and kdr frequency for the F1534C kdr mutation are shown in Table 10. The 1534C mutation was found at high frequency in all populations (>0.6) except at Xaysa-art where the prevalence was low and significantly different than all other populations (pair-wise comparison, P<0.05). Population genetic study showed that all populations were at hardy Weinberg equilibrium (HW, p-value>0.05).

Table 5. Genotype distribution and allelic frequency of the 1534C kdr mutation

Province	Strain	SS	RS	RR	F(R)	Fis	HW(p value)*
Vientiane Capital	Kao-gnot IPL	2	31	85	0.85	-0.0357	1
	Dongpalab	12	43	32	0.62	-0.379	0.818
	Oudomphon	12	43	53	0.69	0.0742	0.505
	Phailom	0	11	87	0.94	-0.0543	1
Luang Prabang	Taling	1	27	88	0.88	-0.0597	1
	Khomkhuang	1	7	100	0.96	0.1929	0.158
	Thongchaleun	2	34	69	0.82	-0.0877	0.511
Saravane	Thatnoy	2	31	84	0.85	-0.0372	1
	Lakhonepheng	0	13	102	0.94	-0.0556	1
Attapeu	Khonsaiy	3	27	20	0.67	-0.2115	0.2
Xayabury	Xaysa-art	37	14	0	0.14	-0.0149	0.575
	Total	**72**	**281**	**720**			

**Hardy Weinberg equilibrium tested by exact-test Fisher. In bold, significant (p-value<0.05) population that showed difference from HW expectations.*

We also check for linkage disequilibrium between V1016G and F1534C in all populations (see Annex 1). Except for the two populations from Khonsaiy and Xaysa-art, there was non-random association of alleles at the two kdr loci (p-value<0.001). The non-independence interaction between kdr mutations may indicate selection process occurring at these two loci.

Genotype-phenotype association

To assess the role of each mutation in permethrin and DDT resistance, we conducted a phenotype–genotype analysis by comparing the genotypic distribution of the V1016G and F1534C mutations between the dead and live mosquitoes after exposure to the two insticides (comparisons were made using Fisher's exact test at 95% CI). The assumption was that a higher proportion of RR genotypes was expected in mosquitoes surviving the insecticide than the ones that died. Considering that the comparisons cannot be done with populations having fixed alleles (i.e. around 1 or 0), we selected populations having kdr allelic frequency for both

mutations ranging from 0.3 to 0.7. Hence only two populations met this criteria, i.e., Oudomphon and Dongpalab.

The results are shown in Table 6. We did not find any significant difference between the frequency of kdr mutations (for both 1534C and 1016G) and the survival rate from DDT and permethrin (p>0.05). This may suggest that the kdr alleles may have no or only a minor role in the phenotype resistance observed to DDT and permethrin. However, the linkage disequilibrium between the two loci may explain this trend as the two loci are not independent from each other. Indeed, we noted negative association between the presence of homozygote-resistant genotypes for 1014G and the ones for the 1534C substitution. In other words, mosquitoes that exhibited RR genotypes for 1016G were mostly SS for the 1534C (and vice versa). This phenomenon may then bias the number of RR genotypes expected in each category (dead or alive). In addition, only two mosquitoes (of 1,076) were found homozygote resistant for both 1534C and 1016G, hence suggesting potential genetic cost associated with the double mutants.

Table 6. V106G and F1534C genotypic differentiation between dead and live mosquitoes for DDT and permethrin

	V1016G				F1534C		
	DDT				**DDT**		
	SS	RS	RR		SS	RS	RR
Alive	27	30	3	Alive	3	30	27
Dead	16	13	6	Dead	5	14	16
p-value	*0.42*			*p-value*	*0.62*		
	Permethrin				**Permethrin**		
	SS	RS	RR		SS	RS	RR
Alive	23	25	8	Alive	11	26	22
Dead	21	15	5	Dead	5	16	20
p-value	*0.47*			*p-value*	*0.27*		

Efficacy of alternative larvicides for dengue vector control in semi-field trial

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Background

To provide information on alternatives to temephos for vector control operations in the Lao PDR, we studied the resistance levels of *Aedes aegypti* mosquitoes from Vientiane to *Bacillus thuringiensis var israeliensis* (*Bti*, bio-insecticide) and the spinosad (naturalyte), each of which has a different mode of action. We also tested the efficacy of the insect growth regulator diflubenzuron (benzoylurea) that acts by disrupting chitin synthesis and deposition (Matsumura 2010). This IGR showed a promising efficacy against several mosquito species, especially *Aedes aegypti*. *Bti* has desirable properties for mosquito control because of its fast killing effect, a good toxicological profile (Lacey 2007), and the absence of cross-resistance with conventionally used pesticides. All of the above insecticides are recommended by the World Health Organization (WHO) for use for vector control in drinking water sources and containers and may be used routinely by mosquito control services (WHO 2004, 2008, 2010).

The purpose of this study was to characterize the resistance status of *Ae. aegypti* larvae from Vientiane, Lao PDR, to temephos, *Bti*, and diflubenzuron and to assess the efficacies and residual activities of these insecticides under simulated conditions.

Materials and methods

The trial was carried out in Vientiane, Lao PDR, on the premises of the Institut Pasteur du Laos. The tents and the plastic containers were installed in early October and the experiment started on the 8th of October (Figure 9). The effects of temephos (1mg/L), diflubenzuron (0.25mg/L), and *Bti* (8mg/L) formulations were evaluated and compared against *Ae. aegypti* larvae (IPL strain). Blue plastic containers with a capacity of 200 liters were used because they are widely used for water storage in Vientiane City and

have been shown to be an important productive breeding habitat for *Ae. aegypti.* These drums were filled with 175 liters of domestic water and covered with a mosquito net to prevent oviposition by wild female mosquitoes in the area and to prevent the deposition of debris. The containers were placed under a shelter to prevent direct exposure to rain and sunlight (Figure 9). All the insecticides were tested at the dosage recommended by the WHO or the manufacturers for the control of mosquito larvae. Twelve containers (three replicates per insecticide) were allocated to insecticides at random. Three were left untreated and used as a control. Groups of 100 third-instar larvae of the F1 generation of the Institut Pasteur du Laos strain (IPL strain collected in sentinel containers at the Institute) were added to each container with one gram of food (dry cat food) at time 0, and then every 10 days. The containers were replenished every 10 days to maintain the initial level of water. Emerging adults were collected from each container by using electric aspirators and then stored at −80°C. Temperature and pH were checked every 10 days with a portable tester to detect any differences between replicate and/or treatments. External temperature and humidity were recorded by using a meteorological unit.

Emergence inhibition rates (% EI) and 95% CIs were calculated for the average of the three replicates per insecticide according to the formula: % EI = ((C – T)/C) × 100 where C is the emergence in the control and T is the emergence in the treated container at the same time period. For each formulation, curves were presented until the % EI decreased to <80%, which corresponded to the threshold generally considered for reapplication of the treatment.

The susceptibility status of the IPL strain against *Bti* and diflubenzuron was measured following the same protocol described above.

Figure 9. Semi field trial at the Pasteur Institute of Laos. Efficacy of conventional versus alternative insecticide formulations used for vector control are tested.

Results

Results of the larval bioassays on the IPL strain (Vientiane capital) are shown in Table 7. The result showed that the IPL strain was fully susceptible to the insecticides *Bti* and Diflubenzuron showing that these insecticides are good candidates for vector control in the Lao PDR.

Table 7: Resistance status of *Aedes aegypti* (IPL strain) against *Bti* and Diflubenzuron

Larvicides	RR_{50}*	RR_{95}*
Bti	0.84	**0.38**
Difflubenzuron	1.1	0.73

**Resistant ratios = LC_{50} of wild strain / LC_{50} of USDA susceptible reference strain.*

The results of the semi-field experiment are presented in Figure 10. Twenty-three weeks after the treatments, the average percentage of emergence in the containers treated with Bti was more than 20%, which is the limit recommended for a new treatment in field conditions. This threshold was exceeded in the containers treated with diflubenzuron after twenty-four weeks, while the temephos treatment showed 96% efficacy (4% emergence) after twenty-eight weeks. One should note the dramatic decrease of emergence in the control containers dropping from 80% to 40% between T110 and T150 (Figure 10). This corresponds to an increasing temperature inside the containers during the same period. During this period, the temperatures rose from 22° C to almost 30° C inside the containers. This event may have consequences also on the treated containers, delaying the increase of emergence for a few weeks.

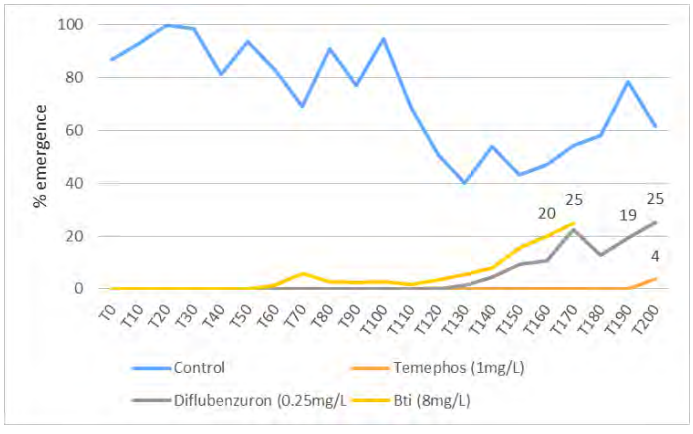

Figure 10. Percentage emergence of *Ae. aegypti* in the control and treated containers

Discussion and Conclusions

The semi-field trial assays showed that after 22 weeks, all the insecticides tested (i.e. *Bti*, temephos, and diflubenzuron) have remained above 80% Emergence Inhibition (EI), i.e. the acceptable threshold for a larvicide according to WHOPES (WHO, 2005). Below 80% EI, a new treatment is recommended. Our results indicated a good residual efficacy of these three insecticide formulations against *Aedes aegypti*. However, it has already been pointed out that the performance of larvicides in well-controlled conditions has to be taken cautiously because of the absence of direct exposure to rain, sunlight, and organic matter that could lead to an over-estimation of the residual activity of an insecticide. Simulated-field experiments represent a useful method for screening new insecticides and/or to select dosages for field application, but they cannot be used to predict the performance of formulated products under real conditions. Despite the relatively good residual activity of the temephos formulation, we should take into account that several populations presented moderate levels of temephos resistance (i.e. RR_{95}>2). Temephos insecticide is the active ingredient of the Abate formulation, the larvicide used for dengue vector control in the Lao PDR. Those levels of insecticide resistance indicate that a selection pressure occurred in these mosquito populations. This emphasizes the need to find a substitute for the Abate formulation to avoid the development of resistance and make future public health operations useless. Furthermore, Abate is posing environmental issues and was banned in the European Union in 2009. The future use of more environmentally friendly products such as Bti and diflubenzuron is recommended, especially knowing that no cross-resistance between these insecticides and temephos has been reported.

The adult bioassays showed that most of the *Ae. aegypti* adult populations were highly resistant to permethrin and DDT, but susceptible to deltamethrin. Permethrin and deltamethrin belong to the pyrethroid family and have been used in the Lao PDR for adult control since the nineties to fight against malaria and dengue. Both have been used in space-spraying applications or in treated bed nets and can be found in household aerosol sprays. This long and intense insecticide use has probably led to the permethrin resistance and deltamethrin resistance is likely to follow soon his long and intense insecticide use has probably led to the permethrin resistance and deltamethrin resistance is likely to follow soon (moderate levels of resistance in larvae, Table 2). Despite the fact that DDT was banned in 1989 in the Lao PDR, strong levels of resistance have been observed. This could be explained by the well-known cross-resistance occurring between pyrethroids and organochlorine (DDT). The use of malathion since the nineties for dengue control certainly explains the high resistance levels in *Aedes* populations in the Lao PDR.

We investigated the presence and the frequency of two well-known kdr mutations (V1016G and F1534C) in 11 field-caught populations of *Aedes aegypti* in the Lao PDR. The results showed that the 1016G and 1534C kdr alleles are present in the Lao PDR but at various frequencies. The 1534C was found at high prevalence (>0.6 except in one population) whereas the 1016G was found at low prevalence (<0.4). In our study no correlation was found between the presence of mutant alleles and the survival rates of mosquitoes to DDT and Permethrin. This can be explained by significant linkage disequilibrium (as discussed above) and/or by the presence of additional mechanisms (metabolic) playing a greater role in resistance than the kdr. Brooke and colleagues (2008) have already showed early showed that resistance could be multigenic, and the kdr genotype might not fully explain all the variance in phenotype. This was confirmed recently by a study published in Genome Research that showed that none of the V1016I/G kdr mutations previously associated with pyrethroid resistance were retained as best candidate markers (Faucon *et al*., 2015). Contradictory findings on the role of the kdr mutations in pyrethroid resistance were previously reported (Saavedra-Rodriguez *et al*., 2007; Donelly *et al*., 2009). Clearly further validation studies are needed to address the exact role of the various kdr mutations in DDT and pyrethroid resistance.

We investigated the role of the detoxification enzymes in the resistance in five resistant populations from Vientiane (2 populations), Luang Prabang, Saravane and Xayabury. Results showed significant differences in detoxification enzyme activities/quantities compared to the susceptible reference strain (USDA) suggesting the involvement of metabolic based resistance mechanisms. Our toxicological data with the use of synergists supported these results. The efficacy of malathion and permethrin against the populations tested was restored after the mosquitoes were exposed to inhibitors of detoxification enzymes. Esterases based-resistance mechanism is a major mechanism for organophosphate resistance in insects (Hemingway and Karunaratne, 1998). Several examples of *Ae. aegypti* resistance to organophosphates worldwide linked to elevated carboxylesterases activities have been described (Rodriguez *et al*., 2001; Wirth *et al*., 1999). Our toxicological and biochemical data confirmed these observations. Among detoxification enzymes, P450s have been shown to play a

major role in pyrethroid resistance in insects (Brogdon *et al*., 1998; Hemingway *et al*., 2004; Ranson & Hemingway, 2005). In the Caribbean, Marcombe *et al*. (2009) suggested the involvement of P450s in the reduced efficacy of deltamethrin space-spray operations. Elevated GST levels have also been frequently associated with insect resistance to insecticides such as DDT and pyrethroids (Vontas et al., 2001; Enayati *et al*., 2005; Lumjuan *et al*., 2005). Our toxicological and biochemical data support the role of P450s and GSTs in insecticide resistance in the Lao PDR.

Better understanding of the levels and mechanisms of insecticide resistance will help to improve the surveillance, the management, and the control of insecticide-resistant dengue vectors in the Lao PDR. The development of new tools such as molecuar biomarkers (i.e. gene Copy Number Variations [CNVs], Single Nucleotide Polymorphisms [SNPs] and differentially expressed genes [DE genes]; Faucon *et al.* 2015) is urgently needed to fight against dengue and to contribute to a fastest, costless and more efficacious control operations in resistant mosquito population areas..

- It is important to maintain a regular monitoring of the levels of insecticide resistance in the different provinces of the Lao PDR to detect the development of insecticide resistance in the dengue vectors
- We recommend the use of alternative insecticides where insecticide resistance is detected
- We recommend to start using new insecticides in rotation (i.e. a different insecticide each year) to avoid or to manage the development of insecticide resistance in the larval and adult populations of the dengue vectors in the Lao PDR

Partners

- U.S. Naval Medical Research Center - Asia
- National Center of Malariology, Parasitology and Entomology (CMPE), Vientiane, Lao PDR
- Kasetsart University, Bangkok, Thailand
- Institut de Recherche pour le Développement, MIVEGEC (UM1-CNRS 5290-IRD 224)

Financial support

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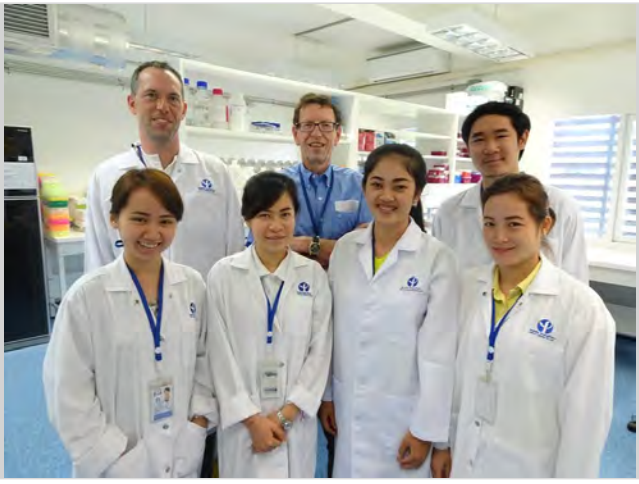
Vaccine Preventable Diseases Laboratory

Lao-Lux joint Lab

The LaoLux Laboratory is operated by the infectious disease research unit at the Luxembourg Institute of Health, Luxembourg (LIH) and aims to build capacity for investigations of important human and animal infectious diseases and to initiate and support collaborative research projects in Lao PDR. The laboratory carries out country-specific research in Lao PDR focusing on vaccine-preventable infectious diseases, zoonotic diseases, identification of new viruses and variants and other investigations. These studies are important to estimate the burden of specific infections, to promote virus outbreak control, to improve animal health welfare and productivity, to support public health policies and vaccination programmes and to optimize health strategies.



Head of Laboratory: Professor Dr Claude P. Muller, Director

Scientists:
Dr. Antony Black, Responsible of the Lab
Dr. Phonethipsavanh Nouanthong
Dr. Keooudomphone Vilivong
Dr. Siriphone Virachith

Technicians:
Ms. Latdavone Khenkha
Mr. Dit Panyathong

Projects

- Serosurveillance of vaccine preventable diseases and hepatitis C in healthcare workers from Lao PDR
- Diphtheria seroprevalence in Huaphan Province
- Hepatitis B and C virus in Lao blood donors
- Varicella zoster outbreak
- Evaluation of zoonotic virus circulation and transmission in domestic animals from mixed farms in rural Lao PDR

The research projects include important components:

- Investigation of public and animal health challenges caused by infectious diseases.
- Training of laboratory and academic staff and students both at IPL and LIH.
- Implementation of new technologies by technology transfer and by providing equipment.
- Providing international visibility to scientists from Lao PDR and access to the international scientific community.
- Dissemination of research results through scientific publications, presentations and international meetings as well as national and international press releases.
- Technical and scientific support for other laboratories in Lao PDR.
- Teaching/training of laboratory staff from collaborating laboratories.

Areas of research and surveillance include:

- Immunology and genetic/antigenic diversity of viruses including molecular epidemiology.
- Public health issues related to infectious diseases in humans (measles virus, rubella virus, mumps virus, hepatitis virus, respiratory viruses etc.).
- Public health and animal welfare issues related to veterinary viruses.

Visiting scientists from Department of Infection and Immunity, Luxembourg:

Dr. Konstantin Evdokimov.
Dr. Maude Pauly

Institut de la Francophonie pour la Medecine Tropicale students trained in 2015:

Dr. Kinnaly Saidalasuk (Junior Scientist since October 2015).
Dr. Vilaysone Khounvisith (Junior Scientist since October 2015).
Dr. Phonepaseuth Khampanisong (Junior Scientist since October 2015).

Financial support

The laboratory is funded by a grant from the Government of the Grand Duchy of Luxembourg.

Serosurveillance of vaccine preventable diseases and hepatitis C in healthcare workers from Lao PDR

Project coordinator: Antony Black
Member of staff: Keooudomphone Vilivong

Background

Healthcare workers (HCW) have increased risk of exposure to infectious diseases and infected HCW represent a potential source for onward transmission of pathogens to susceptible patients. It is important that these risks are minimized, both by reducing exposure and by vaccination in the case of vaccine-preventable infections. In the Lao PDR, the 18,017 HCW (2005), corresponded to a ratio of only 3.21 per 1000 population. Whilst there are no data on the immunity of Lao HCW against important infectious diseases, exposure is probably high considering that the lack of resources undermines vaccination coverage and post-vaccination follow-up for this important risk population. In addition, low rates of childhood vaccination within the country and waning of vaccine-induced antibodies in adults further exacerbate their risk.

Many countries advise that HCW should receive 3 doses of HBV vaccine. Furthermore, if there is no evidence for immunity to measles, mumps and rubella, 2 doses of vaccine are recommended. Similarly, a booster for diphtheria, tetanus and pertussis and varicella vaccine should be given to non-immune HCW. Other recommendations include management of risk of infection by increased awareness and protection. Nevertheless, in Lao PDR there is currently no national policy with respect to immunisation or serological screening of Lao HCW.

The objective of this cross-sectional seroprevalence study was to determine the level of vaccine coverage, exposure and risk of infection in Lao HCW against HBV, HCV, measles, rubella, tetanus, diphtheria, and varicella zoster.

Activities

1128 HCW were recruited from 3 central, 2 provincial and 8 district hospitals within Vientiane Capital, Huaphan and Boulhikhamxay provinces in Lao PDR. Following informed consent, 9ml of venous blood was collected and age, sex, length of time in the job, job description and numbers of children and self-reported vaccination history were recorded. All participants gave their written informed consent. Results from the HBV and HCV serological testing were reported to the participants and anonymous data were shared with the hospital management according to the informed consent. The study was authorized by the Lao National Ethics Committee for Health Research.

Table 1. Age-stratified seroprevalence of HCW. Anti-diphtheria, anti-tetanus and anti-HBs represent protective levels of antibodies.

	Age n (%)								
	15–24	**25–29**	**30–34**	**35–39**	**40–44**	**45-49**	**50–54**	**55–70**	**Total**
Anti-HBs+	60 (43.2)	127 (50.4)	67 (53.6)	53 (52.5)	67 (52.8)	95 (60.9)	67 (58.3)	31 (56.4)	567 (53.0)
HBsAg+	6 (4.3)	16 (6.3)	5 (4.0)	13 (12.9)	18 (14.2)	9 (5.8)	13 (11.3)	6 (10.9)	86 (8.0)
Anti-HBc+	56 (40.3)	98 (38.9)	59 (47.2)	49 (48.5)	78 (61.4)	81 (51.9)	70 (60.9)	32 (58.2)	523 (48.8)
Anti-HCV+	4 (2.8)	5 (1.9)	1 (0.7)	2 (1.9)	10 (7.4)	8 (4.9)	8 (6.8)	6 (10.5)	44 (3.9)
Anti-measles+	125 (93.3)	233 (95.9)	123 (96.1)	95 (93.1)	121 (95.3)	152 (96.8)	107 (94.7)	55 (98.2)	1011 (95.4)
Anti-rubella+	109 (81.3)	194 (79.5)	120 (93.8)	88 (85.4)	113 (87.6)	143 (89.9)	99 (87.6)	53 (94.6)	919 (86.2)
Anti-diphtheria+	59 (43.7)	110 (44.2)	71 (55.0)	46 (44.2)	88 (67.7)	110 (69.2)	75 (64.7)	37 (66.1)	598 (55.6)
Anti-tetanus+	115 (84.6)	216 (86.7)	103 (81.1)	72 (69.2)	110 (84.6)	121 (76.1)	79 (68.7)	31 (55.4)	847 (78.7)
Anti-varicella+	127 (94.1)	228 (91.2)	122 (94.6)	102 (98.1)	126 (96.9)	155 (96.3)	113 (97.4)	54 (96.4)	1027 (95.0)

Only 53.1% of the HCW had protective anti-hepatitis B surface antigen antibodies with 48.8% having anti-hepatitis B core antibodies, indicating previous exposure and 8.0% were hepatitis B surface antigen carriers. 3.9% were hepatitis C seropositive. Measles and rubella antibodies were detected in 95.4% and 86.2% of the HCW, with 11.9% of females

being unprotected against rubella. Antibodies against varicella zoster, tetanus and diphtheria were detected in 95%, 78.8% and 55.3%, respectively (Figure 1). Seroprevalence varied according to age, gender and number of children (Table 1).

Our data show that an unacceptably high proportion of Lao HCW remain susceptible to infection with hepatitis B, diphtheria, tetanus and rubella. Some public health investigators recommend serological testing of HCW prior to employment. However, this is not feasible in Lao PDR. Thus, considering scarce resources, we recommend systematic vaccination of Lao HCW whenever the vaccine is available. Furthermore, the high prevalence of chronic hepatitis B and C in HCW suggests that risk management strategies should be enforced to protect both HCW and patients.

Finally, the HCW attitude towards their own vaccination is likely to mirror their permissive attitude to vaccination in general. Therefore, vaccination awareness campaigns for HCW in hospitals would be of very broad benefit. As a minimum, a risk awareness strategy is warranted in this context in addition to 3 dose HBV vaccination for all Lao HCW, irrespective of vaccination history.

Reporting

These data have been reported to the Lao MOH.

Manuscript:

Black AP, Vilivong K, Nouanthong P, Souvannaso C, Hubschen JM, et al. (2015) Serosurveillance of Vaccine Preventable Diseases and Hepatitis C in Healthcare Workers from Lao PDR. PLoS One 10: e0123647.

Partners

- National Center for Laboratory and Epidemiology, Vientiane, Lao PDR.
- Vientiane central hospitals; Mitthapab, Settathirath and Childrens.
- Vientiane district hospitals; Sykhod, Chantabouly, Sysatanak, Xaysetha.
- Provincial Lao hospitals; Huaphan and Boulhikhamxay.
- National Immunization Programme, Ministry of Health, Lao PDR.

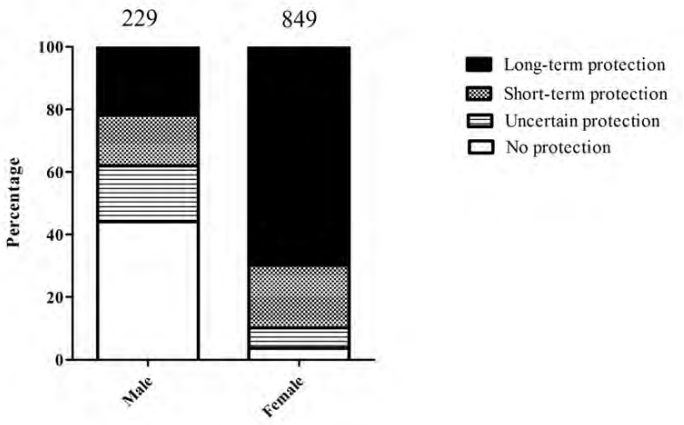


Figure 1. Tetanus antibodies according to gender of HCW. Numbers above columns represent number per group.

Diphtheria seroprevalence in Huaphan Province.

Project coordinator: Antony Black
Member of staff: Keooudomphone Vilivong, Phonethipsavanh Nouanthong

Background

Diphtheria is a vaccine-preventable infectious disease caused by the bacterium *Corynebacterium diphtheriae*. The disease is spread primarily by droplets from the nose, throat and eyes and affects all ages but is more prevalent in unvaccinated individuals below the age of 15 years. Between October and mid-December 2012, the National Centre for Laboratory and Epidemiology (NCLE) reported 93 suspected cases of diphtheria, including 6 deaths, from the Xamtai and Huameuang districts, Huaphan province. Age distribution was specified for 24 suspected cases (29.2% under 4 years, 41.7% between 4 and 9 years, 20.8% between 10 and 14 years, 8.3% older than 14 years). Further outbreaks occurred in other provinces and continued in 2013 (about 29 suspected cases in Huaphan and 20 more nationwide in 2013). Such reemergence of a serious but vaccine-preventable disease could be due either to poor vaccination

coverage or low effectiveness of the vaccines used.

In collaboration with the Institut de la Francophonie pour la Medecine Tropicale in Vientiane, we aimed to assess the diphtheria immunization status of children in two districts of the province of Huaphan and to determine the reasons for non-vaccination and lack of seroconversion among vaccinated children.

Following parental informed consent, 132 blood samples were taken from children between the ages of 12 to 59 months in Kuan and Xamtai districts of Huaphan province. Factors associated with non-vaccination are shown in Table 2. Antibodies to Diphtheria toxin were detected in 84 children (63.6%) of which 58 (43.9%) had a titer greater than 1 international unit per milliliter (IU/ml) corresponding to a long-term protection. Children from villages within 100 minutes of a health center were significantly better immunized against diphtheria ($p < 0.001$) than the children of more remote villages. Tetanus antibodies were also higher in those villages closer to health centres (Figure 2). We also found a high percentage (65%) of the children had no protective antibodies against hepatitis B virus and 49% were infected with intestinal parasites. These data indicate very low vaccine coverage and high intestinal parasite infections in Huaphan province, particularly in rural areas far from health centres. The occurrence of a diphtheria epidemic in the Huaphan Province clearly reveals a serious deficiency in immunization coverage of children under 5 years of age. Whether significant proportions of children targeted by the EPI are not reached by mobile teams or the vaccines are poorly preserved and used, or that the immunogenicity may have been low, remains to be determined more precisely but the first two factors appear to be associated predominantly.

This study supports the findings of previous surveys conducted in Lao PDR leading to the following recommendations: strengthening health education for target populations, prioritization of vaccination in the national health strategies, reinforcement of mobile teams in remote areas, improving coordination with village leaders to reach all target children, strengthening of equipment of all health centers (fridges, kerosene and/or gas), ensuring regular supply of vaccines, cold chain management and adoption of temperature-monitoring technologies during storage and transport of vaccines. With regard to ethnic minorities, an anthropological approach should be implemented to take into account any cultural barriers to vaccination. The implementation of these recommendations should be a priority in the most remote provinces in order to avoid the recurrence of outbreaks of deadly but vaccine preventable diseases in children.

Prospective

These data were presented in poster format at the National Health Research Forum, Vientiane, October 2013 and have been reported to the Lao MOH.

Manuscript

Nanthavong N, Black AP, Nouanthong P, Souvannaso C, Vilivong K, et al. (2015) Diphtheria in Lao PDR: Insufficient Coverage or Ineffective Vaccine? PLoS One 10: e0121749.

Partners

- Institut de la Francophonie pour la Médecine Tropicale.
- Laos-Japan Joint Laboratory for Parasitology, Institut Pasteur du Laos, Vientiane, Lao PDR.

Table 2: Logistical regression; factors associated with non-vaccination of children. ref: reference; cOR: crude Odds ratio; aOR: adjusted Odds ratio; 95% CI: 95% confidence interval.

Independent variables	Bivariate analysis			Multivariate analysis		
	cOR	95% CI	p	aOR	95% CI	p
Father's educational level						
Illiterate (ref)	-	-	-	-	-	-
Primary	0.85	[0.24-2.95]	0.7	-	-	-
Mother's educational level						
Illiterate (ref)	-	-	-	-	-	-
Primary	0.08	[0.01-0.63]	0.01	0.08	[0.008-0.81]	0.03
Ethnic group						
Laoloum (ref)	-	-	-	-	-	-
Laosoung	11	[2.2-54.9]	0.003	1.73	[0.23-13.18]	0.5
Laotheung	16	[2.8-92.2]	0.002	12.2	[1.74-85.4]	0.01
Religion						
Buddhist (ref)	-	-	-	-	-	-
Animist	5.7	[0.72-45.3]	0.09	7.59	[0.36-159.7]	0.2
Time to reach the nearest health center (minutes)						
<100 (ref)	-	-	-	-	-	-
>100	2.04	[0.69-6.02]	0.1	6.35	[1.4-28.8]	0.01
Sex of child						
Male (ref)	-	-	-	-	-	-
Female	0.78	[0.26-2.32]	0.6	-	-	-
Advice on immunization received at birth						
Yes (ref)	-	-	-	-	-	-
No	9.7	[2.6-36.8]	0.001	9.8	[1.5-63.8]	0.01

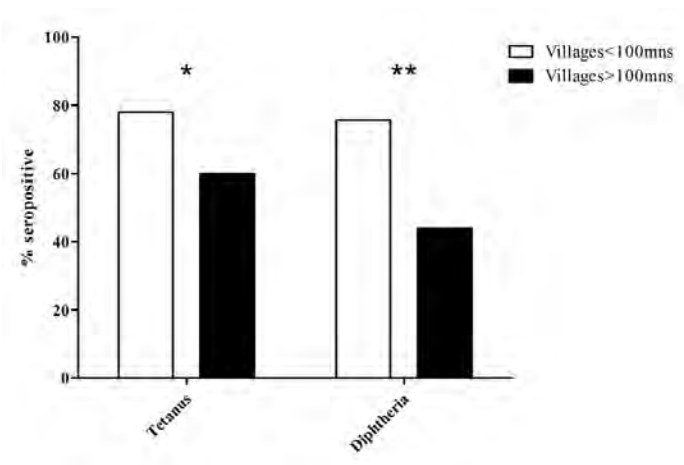
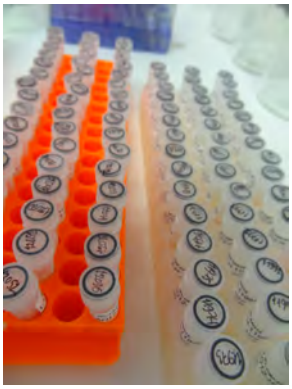


Figure 2. Anti-diphtheria and anti-tetanus antibody seroprevalence in children from villages less than or greater than 100 minutes travel from health centres (n = 132; mns = minutes; *p = 0.02, **p<0.001).

Hepatitis B and C virus in Lao blood donors.

Project coordinator: Antony Black
Member of staff: Phonethipsavanh Nouanthong



Background

Hepatitis B virus (HBV) is highly endemic in the Lao People's Democratic Republic with up to 10% of blood donors being positive for HBsAg. Luxembourg Institute of Health, Luxembourg and the Lao Red Cross have also previously found a high prevalence of occult infection in Lao blood donors. Such potentially infectious blood is currently not screened for in the Lao blood donations. The current study aims to expand these findings in a nation-wide survey.

Activities

The HBV serology profile was determined among approximately 5000 first-time and repeated donors during 2014-2015 in six representative provinces.

Preliminary results

Prevalence of HBsAg was high in first time Lao blood donors. Repeat donors also had high prevalence, suggesting that they have not been effectively screened out of the donation process. Repeat donations from HBsAg positive donors need to be reduced, in order to save cost and resources.

Prospective

The data were presented and discussed with the Lao Red Cross. The implementation of an effective database tracking system during blood donor recruitment could be a cost-effective way to exclude repeat donors that were previously tested positive.

Partners

- Lao Red Cross, National Blood Transfusion Centre, Vientiane, Lao PDR.
- Department of Pathology, University of Health Sciences, Vientiane, Lao PDR.
- Department of Pathology, Faculty of Medicine, Chiang Mai University, Thailand.

Varicella zoster outbreak

Project coordinator: Antony Black
Member of staff: Phonethipsavanh Nouanthong

Background

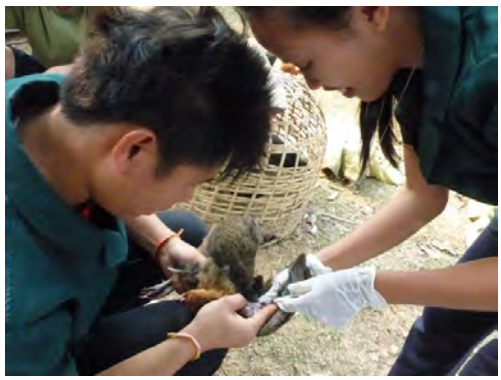
The National Centre for Laboratory and Epidemiology (NCLE) was notified regarding an outbreak situation in a remote region of Parkseng district, Luang Prabang Province in January 2015. An investigation team included epidemiologists and laboratory scientists from NCLE, WHO, and Institute Pasteur du Laos. Clinical diagnosis was performed by a physician and blood samples from all subjects with clinically diagnosed VZV infection were collected by the IPL scientist and clinician. Suspected cases were tested for presence of anti-VZV IgM at IPL. Anti-VZV IgM was detectable in 30 out of 34 cases (88.2%) with an average age of 6.5 years. These data confirmed the outbreak was varicella zoster and, together with nationwide serology data (not shown) indicate that the virus is widely circulating in Lao PDR.

Prospective: all data were reported to NCLE.

Photo: IPL staff collecting and organising samples from varicella outbreak.

Evaluation of zoonotic virus circulation and transmission in domestic animals from mixed farms in rural Lao PDR

Project coordinator: Maude Pauly



Background

Together with sub-Saharan Africa, South-East Asia represents a major hotspot for emerging infectious diseases, largely due to close contacts between wildlife, domestic animals and humans. People in rural areas of Lao PDR are predominantly subsistence farmers and cultivators and Lao farmers practice mixed-species backyard farming, where poultry, pigs, ruminants and other animal species are traditionally reared together. Livestock is reared mostly for direct consumption, but also as potential cash reserve or as “wealth insurance”. Thus, animal health directly influences human well-being, since the loss of an animal entails not only loss of protein provision, but also of the cash reserve required in emergency situations (e.g., a medical treatment). Despite the considerable risk for interspecies and zoonotic disease transmission, human and animal health care services are insufficient and early and rapid detection of disease outbreaks remains challenging.

Activities:

In collaboration with the local partners, samples from domestic animals (including cattle, small ruminants, poultry and pigs) were collected in rural settings and in slaughterhouses and tested for several relevant viral animal diseases (e.g. Newcastle disease, Infectious bronchitis virus) and for viruses with zoonotic potential (e.g. Influenza A, Hepatitis E, Paramyxoviruses, Coronaviruses) applying different laboratory techniques. Particular focus was put on

close collaboration with people who are in contact with local farmers on a daily basis to increase the chance to detect and control disease outbreaks. Hereby, we aim to provide our partners with the required capacity and knowledge for disease outbreak management. They were involved in all steps of study design, sample collection, processing and analyses. Moreover, knowledge transfer was assured by organizing workshops on basic laboratory analyses, by actively participating in on-site sample collection and by giving lectures (e.g. on basic virology, on zoonotic diseases).

Partners

- Faculty of Agriculture, University of Laos, Lao PDR.
- Veterinarians without borders.
- Department of Pathology, University of Health Sciences, Vientiane, Lao PDR.
- Department of Pathology, Faculty of Medicine, Chiang Mai University, Thailand.
- National Animal Health Laboratory, Vientiane, Lao PDR.

Parasitology Laboratory

Lao-Japan joint Lab

Aims of the Parasitology laboratory are to carry out research and training in the area of parasitology to better understand parasitic diseases affecting the Lao population and to propose ways to mitigate possible infections, and to provide technical support to the national level institutions in the area of malaria and other parasitic diseases.

Head of Laboratory: Shigeyuki KANO, MD, Ph D

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Projects

- SATREPS Project: Project for Development of Innovative Research Technique in Genetic Epidemiology of Malaria and Other Parasitic Diseases in Lao PDR for Containment of Their Expanding Endemicity

Project for Development of Innovative Research Technique in Genetic Epidemiology of Malaria and Other Parasitic Diseases in Lao PDR for Containment of Their Expanding Endemicity

Project coordinator: Dr. Shigeyuki Kano
Member of staff: Moritoshi Iwagami, Phonepadith Xangsayarath, Phonepadith Khattignavong, Sengdeuane Keomalaphet, Pheovaly Soundala, Lavy Lorphachan, and other visiting scientists and students

Background

Malaria, Schistosomiasis (*Schistosoma mekongi*) and Opisthorchiasis (*Opisthorchis viverrini*) have tremendous health burden on the people in Lao PDR. Although significant reductions in malaria transmission have been reported due to the large-scale insecticide-treated bed nets (ITNs) distribution through the Global Fund to Fight AIDS, Tuberculosis and Malaria, strategies based on the scientific evidence have not been developed to deal with the genetic variation in parasites and vectors population, and drug resistant malaria. Recently, artemisinin resistant malaria was reported in Attapeu province in 2014 (Ashley et al., 2014). Therefore, it is necessary to survey other provinces especially in the southern part of the country to monitor and contain the spread of drug resistant malaria. Lao Ministry of Health and WHO set a goal to eliminate malaria by 2030. To achieve this goal, we have to understand real malaria situation including drug resistant malaria and to develop elimination strategies.

Since Schistosomiasis (*S. mekongi*) and Opisthorchiasis (*O. viverrini*) are localized to Lao PDR and surrounding countries, they are recognized as neglected tropical diseases. Nevertheless, the prevalence of Opisthorchiasis is estimated as high as 15-54% in Lao PDR. Little information on the molecular/genetic epidemiology of the Opisthorchiasis is available to develop effective measures for prevention and diagnosis of the diseases.

The Government of Lao PDR requested Japan International Cooperation Agency (JICA) to establish the Lao-Japan Joint laboratory within Institut Pasteur du Laos (IPL) for conducting highly technological research on human malaria parasites: *Plasmodium falciparum*, *P. vivax*, and monkey malaria parasite: *P. knowlesi*, and human trematodiasis: *S. mekongi* and *O. viverrini*. The joint research will concentrate on genetic epidemiological studies to detect and control of the emergence and dissemination of these parasitic diseases. The Project also contributes to the capacity development of researchers and technicians in Lao PDR through training of field and Lab work, seminar and career development.

In order to carry out this project, the IPL collaborates with National Center for Global Health and Medicine (NCGM), Tokyo, Japan, Center of Malariology, Parasitology and Entomology (CMPE), National Institute of Public Health (NIOPH), and other Departments, Ministry of Health, Lao PDR.

Objective

Objectives of this project are (1) to develop more convenient and accurate methods (PCR methods, LAMP methods, etc.) for diagnosis of the diseases, (2) to monitor temporal and spatial epidemiological situations of pathogens and vectors of the diseases, (3) to analyze mechanisms of emergence and expansion of the drug resistant malaria, especially, artemisinin resistance, and (4) to analyze glucose-6-phosphate dehydrogenase (G6PD) activity of Lao population for evaluation of possible usage of primaquine (Howes et al., 2013), utilizing molecular biological techniques.

Based on the scientific evidence obtained by this project, health education for the people will be strengthened and endemicity of the diseases will be monitored together with the local Lao Ministry of Health. Research results will also be utilized in government services for sustainable development of Lao PDR.

Study period of the project

Five years (May 2014 to April 2019)

Study sites of the project

Malaria:

Savannakhet province, Saravane province, Sekong province, Attapeu province, Champasak province, Khammouane province, Phong Sali province, Luang Prabang province

Schistosomiasis (*S. mekongi*):
Khong district and Mounlapamok district, Champasak province

Opisthorchiasis (*O. viverrini*):
Khammouane province, Champasak province

Ethical clearance

The SATREPS project was approved by the National Ethics Committee for Health Research in the National Institute of Public Health (NIOPH), Ministry of Health, Lao PDR in 2014, 2015 (extend every one year).

Activities and Results in October 2014 to October 2015

We conducted 7 field surveys on parasitic diseases and 3 training courses of parasitic diseases diagnoses for medical lab technicians at IPL and 5 training courses in 5 southern provinces (Savannakhet, Saravane, Sekong, Champasak, and Attapeu) since we published IPL Activities Report 2014. This activities report summarized our activities and results of the SATREPS project in Parasitology lab from October 2014 to October 2015.

All field surveys were conducted with collaboration with Center of Malariology, Parasitology and Entomology (CMPE), Provincial Health Office, and District Health Office, Ministry of Health, Lao PDR. All the training courses at IPL were also conducted with collaboration with CMPE and all the training courses at provinces were conducted with collaboration with CMPE and Provincial Health Office.

Field surveys on Malaria, Schistosomiasis and Opisthorchiasis

- Schistosomiasis and Opisthorchiasis Survey, Khong district, Champasak province on 8th-12th December 2014
- Malaria Survey of foreign migrant population, Thapangtong district, Savannakhet province on 26th February-5th March 2015
- Schistosomiasis and Opisthorchiasis Survey, Khong district, Champasak province on 20th-25th April 2015
- Malaria Survey, Xaysetha district, Phouvong district, Sanamxay district, Attapeu province on 9th-16th May 2015
- Schistosomiasis and Opisthorchiasis Survey, Mounlapamok district, Champasak province on 17th-23rd May 2015
- Opisthorchiasis Survey, Yommalath district, Khammouane province on 16th-26th August 2015
- Opisthorchiasis Survey, Yommalath district, Khammouane province on 20th-30th September 2015

Malaria field surveys

Written informed consent was obtained from all the participants prior to the interview and blood collection for malaria diagnosis. Three malaria diagnostic methods: microscopy, rapid diagnostic test (RDT: Malaria Ag *Pf/Pv,* Standard Diagnostics, Inc. Republic of Korea) and DNA diagnostic test (PCR method) were used for detecting malaria parasites, malaria parasites antigen and malaria parasites DNA, respectively.

Malaria Survey for foreign migrant population, Thapangtong district, Savannakhet province on 26th February - 5th March 2015

This survey was conducted in collaboration with Dr. Tiengkham POMGVONGSA, Director of Malaria Section, Savannakhet Provincial Health Office, and a team of University of the Ryukyus leaded by Professor Jun KOBAYASHI, Associate Professor Daisuke NONAKA, who are members of the SATREPS project. Seventy-seven foreign migrant workers participated in this malaria survey: 64 Vietnamese, 7 Chinese and 6 Thai. Four out of 77 participants refused blood examination (malaria RDT & microscopy) and blood collection on a filter paper. Malaria RDT was conducted on site of the survey, while microscopy was conducted at Malaria Station in Savannakhet province, and PCR analysis was conducted at IPL. All the participants were malaria negative by all the diagnostic methods.

Malaria Survey, Xaysetha district, Phouvong district, Sanamxay district, Attapeu province on 9th-16th May 2015

A master course student, Dr. Sengdeuane KEOMALAPHET, from Institut Francophone de Medecine Tropicale (IFMT) joined this survey and now she is a member of Parasitology lab at IPL. A total of 719 villagers in Attapeu province participated in this malaria survey. Table 1 shows summary of the survey.

Table 1. Summary of Malaria survey in Attapeu province in May 2015

District	Village	Population	No. of Participant	RDT	
				Pf	*Pv*
Xaysetha	Don Ngew	903	132	0	0
	Hard Sun	1053	120	0	0
Phouvong	Vong Say	431	107	1	1
	Ta Oum	330	104	0	0
Sanamxay	Hard Sai Soung	280	102	0	0
	Som Poi	1020	154	1	2
Total			**719**	**2**	**3**

RDT: Rapid Diagnostic Test (Malaria Ag *Pf* / *Pv*, Standard Diagnostics, Inc. Republic of Korea), *Pf* : *Plasmodium falciparum*, *Pv* : *P. vivax*

RDT was conducted on site of the survey, and microscopy and PCR analysis were conducted at IPL. When the participants were diagnosed as malaria by RDT in the survey, Health Center or District Hospital provided antimalarial drugs (Coartem: artemether and lumefantrine) to them. A total of 381 blood samples (120 from Hard Sun, 107 from Vong Say and 154 from Som Poi villages) were analyzed by the 3 methods (Table 2). The remaining 338 blood samples are under analysis at IPL. Genomic DNA was extracted from the filter papers using a QIAamp DNA Mini Kit® (Qiagen, Valencia, CA, USA). Nested real-time PCR was used to detect malaria parasites DNA. First, universal human malaria parasites primer set was used, and second, species specific primer sets (*P. falciparum* and *P. vivax*) were used for malaria parasites DNA detection. Partial *cytochrome b gene* in mitochondrial genome of *P. falciparum* and *P. vivax* was used as a target of the PCR analysis. A total of 339 samples were malaria DNA negative, while 42 samples were malaria DNA positive by real-time PCR methods. Table 2 shows detail of 42 malaria DNA positive cases in this study.

All the 42 malaria parasites DNA positive cases were asymptomatic. Average body temperature of them was 36.7°C and no one was higher than 37.5°C. Average age of them was 29 years old. Number of male and female were 36 and 6, respectively ($P < 0.05$). A manuscript of these data is in preparation for publication.

Table 2. Summary of malaria positive cases by 3 diagnostic tests

District	village	Age	Sex	Body tem.	Microscopy		RDT		Real-Time PCR	
					P.f	P.v	P.f	P.v	P.f	P.v
Saysetha	Hard Sun	60	M	36.6						+
Saysetha	Hard Sun	48	M	36.9						+
Phouvong	Vong Say	27	M	36.5	+		+		+	
Phouvong	Vong Say	26	M	36.0		+		+		+
Phouvong	Vong Say	35	M	36.4						+
Phouvong	Vong Say	29	F	36.9					+	
Phouvong	Vong Say	23	F	36.7						+
Phouvong	Vong Say	45	F	36.3						+
Phouvong	Vong Say	30	M	36.4						+
Phouvong	Vong Say	25	F	36.5					+	
Phouvong	Vong Say	24	M	36.6						+
Phouvong	Vong Say	47	M	37.1						+
Phouvong	Vong Say	41	M	36.7					+	
Phouvong	Vong Say	14	M	36.9						+
Sanamxay	Som Poi	22	M	36.9						+
Sanamxay	Som Poi	23	M	36.6		+		+		+
Sanamxay	Som Poi	34	M	36.7						+
Sanamxay	Som Poi	22	M	36.3						+
Sanamxay	Som Poi	22	M	36.7						+
Sanamxay	Som Poi	14	M	37.0						+
Sanamxay	Som Poi	15	M	36.7						+
Sanamxay	Som Poi	21	F	36.9						+
Sanamxay	Som Poi	22	M	36.9			+		+	
Sanamxay	Som Poi	30	M	36.8						+
Sanamxay	Som Poi	18	M	36.8				+		+
Sanamxay	Som Poi	24	M	37.1						+
Sanamxay	Som Poi	30	M	36.5						+
Sanamxay	Som Poi	29	M	36.6						+
Sanamxay	Som Poi	42	F	36.8						+
Sanamxay	Som Poi	16	M	36.2						+
Sanamxay	Som Poi	17	M	37.0						+
Sanamxay	Som Poi	41	M	36.3						+
Sanamxay	Som Poi	13	M	36.7						+
Sanamxay	Som Poi	23	M	36.6						+
Sanamxay	Som Poi	46	M	36.8						+
Sanamxay	Som Poi	24	M	36.4						+
Sanamxay	Som Poi	36	M	36,7						+
Sanamxay	Som Poi	57	M	36.7						+
Sanamxay	Som Poi	33	M	36.9						+
Sanamxay	Som Poi	20	M	36.8						+
Sanamxay	Som Poi	15	M	36.9						+
Sanamxay	Som Poi	35	M	36.9						+

Discussion of malaria survey in Attapeu province in May 2015

The analyses showed that all of the positive cases were asymptomatic malaria parasites carriers (parasite reservoir, or hidden malaria) and that adult male was a high-risk population in the areas. According to WHO World Malaria Report 2015, 62% of malaria cases in Laos were *P. falciparum*

and 38% of them were *P. vivax*. On the contrary, our survey showed that *P. vivax* is a dominant species (88.1%: 37/42) in the study areas. Our survey was conducted in May (dry season). This might be influenced malaria transmission dynamics in the endemic area. Generally, malaria season is rainy season because mosquito density is high in rainy season, although this pattern is getting unclear in Laos. *P. vivax* has a dormant stage called "hypnozoite" in human liver cells that causes relapse of vivax malaria months, or even a year later. Only primaquine can treat hypnozoite but this antimalarial drug cannot be used for people who have G6PD deficiency. G6PD deficiency is a genetic disorder and if G6PD deficiency people take primaquine, acute hemolytic anemia is liable to occur. In Laos, this drug is not officially used because few information about G6PD deficiency among the population is available so far and G6PD test kit is not available in the country yet, except Luang Prabang, Savannakhet and Champasak provinces where a pilot primaquine treatment for vivax malaria patients was conducted in 2015 by Lao Ministry of Health with support of WHO. Therefore, *P. vivax* has a potential to be a dominant species during dry season in Attapeu province.

Further survey, especially in rainy season is needed to understand malaria transmission in the study areas. Moreover, to understand real malaria transmission status in the country, wide range of malaria sample collection in 5 southern provinces (Savannakhet, Saravane, Sekong, Champasak, and Attapeu) has been conducted by the SATREPS project with the collaboration of Provincial Health offices, District Hospitals and Health Centers.

Schistosomiasis and Opisthorchiasis Surveys

Written informed consent was obtained from all the participants prior to the interview and stool collection for diagnosis of Schistosomiasis and Opisthorchiasis.

Schistosomiasis and Opisthorchiasis Survey, Khong district, Champasak province on 8th-12th December 2014

This survey was conducted at Donetharn Secondary School, Donlong Island in Khong district. It was one week before mass drug administration (MDA) of praziquantel. One hundred and ninety-two students participated in this survey. Stool examination by microscopy was conducted by 2 methods: Kato-Katz (KK) method and Formalin-Detergent (FD) method for detecting the parasite eggs in the stool samples either on site or at IPL. The results of the stool examination were shown in Table 3 (KK method) and 4 (FD method). By KK method, 25.0% of students (48/192) were *O. viverrini* positive, while by FD method, 25.7% of students (49/191) were *O. viverrini* positive including mixed infection with *O. viverrini* and other parasites. *S. mekongi* egg was not observed in this study.

Table 3. Results of KK method in Khong district, Champasak province in December 2014

Parasites	No.	%
Opisthorchis viverrini (Ov)	38	19.8
Hookworm	25	13.0
Taenia	1	0.5
Enterobius	1	0.5
Ov + Hookworm	8	4.2
Ov + Trichostrongylus	1	0.5
Ov + Enterobius	1	0.5
Hookworm + Taenia	1	0.5
Negative	116	60.4
Total	**192**	**100.0**

Table 4. Results of FD method in Khong district, Champasak province in December 2014

Parasites	No.	%
Opisthorchis viverrini (Ov)	41	21.5
Hookworm	25	13.1
Trichuris trichiura	1	0.5
Taenia	1	0.5
Giardia cyst	1	0.5
Ov + Hookworm	5	2.6
Ov + Giardia cyst	2	1.0
Ov + Hookworm + Giardia cyst	1	0.5
Negative	114	59.7
Total	**191**	**100.0**

DNA diagnostic method (LAMP method) was also conducted using the 110 stool samples at Tokyo Medical and Dental University, Japan. Only 7 out of 110 samples were *Opisthorchis viverrini* DNA positive by LAMP method.

A total of 2,559 freshwater snails (resembling *Neotricula aperta*) was collected at Donglong Island near the school. Infection rate of *S. mekongi* of the snails was examined by microscopy and by LAMP method targeting *cox1* gene of mitochondrial genome of *S. mekongi*, but all of the snails were negative for *S. mekongi.*

Schistosomiasis and Opisthorchiasis Survey, Khong district, Champasak province on 20th-25th April 2015

This survey was conducted at Donetharn Secondary School, Donlong Island in Khong district 4 months after MDA of praziquantel. One hundred and fifty-two students participated in this survey. They are same population at the same school in the previous survey in December 2014. However, the sample size of this survey (152) was smaller than that of the previous survey (192) because some of the students were taking Lao new year holidays (middle of April). Stool examination by microscopy was conducted by Kato-Katz (KK) method (triplicate) for detecting parasite eggs in the stool samples either on site or at IPL. The results of stool examination were shown in Table 5. In this survey, 21.1% of students (32/152) were *O. viverrini* positive including mixed infection with *O. viverrini* and other parasites, while 1.3% of students (2/152) were *S. mekongi* positive. They are mixed infection with *O. viverrini.*

Table 5. Results of KK method (Triplicate) in Khong district, Champasak province in April 2015

Parasites	No.	%
Opisthorhcis vierrini (Ov)	24	15.8
Hookworm	13	8.6
Ov + Hookworm	6	3.9
Ov + *S. mekongi*	2	1.3
Enterobius	1	0.7
Trichuris trichiura	1	0.7
Hookworm + Taenia	1	0.7
Negative	104	68.4
Total	**152**	**100.0**

DNA diagnostic method (LAMP method) was also conducted using the 152 stool samples at Tokyo Medical and Dental University, Japan. ITS1 region of rRNA gene of the parasites nuclear genome was used as a target gene for LAMP method. 13.8% of samples (21/152) were *Opisthorchis viverrini* DNA positive, while 0.7% of samples (1/152) were *S. mekongi* DNA positive by LAMP method. This result suggested that sensitivity of LAMP method was lower than that of microscopy (KK method) or that the *O. viverrini* egg might be an *O. viverrini*-like egg, "minute intestinal fluke" (Sayasone et al., 2009). Further analysis is needed to elucidate this issue. A manuscript of these data is in preparation for publication.

A total of 1,975 freshwater snails (resembling *Neotricula aperta*) was collected at Donglong Island near the school. Infection rate of *S. mekongi* in the snails was examined by microscopy and LAMP method targeting *cox1* gene of mitochondrial genome of *S. mekongi*, but all of the snails were negative for *S. mekongi*. Species of the collected snails was identified by PCR and DNA sequencing in Tokyo Medical and Dental University, Japan, as *Neotricula aperta* gamma strain which is reported as a host of *S. mekongi*. The molecular phylogenetic tree of the snails was shown in Figure 1.

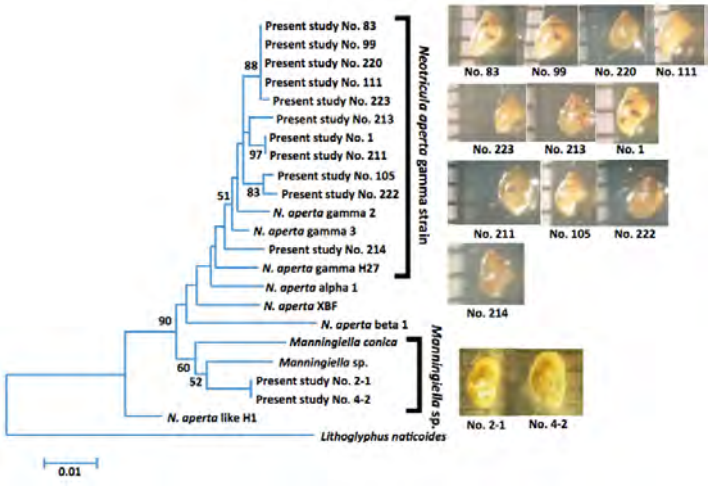

Figure 1. Molecular phylogenetic tree of *Neotricula aperta* gamma strain collected in Donglong Island in Champasak province in 2015

Schistosomiasis and Opisthorchiasis Survey, Mounlapamok district, Champasak province on 17th-23rd May 2015

This survey was conducted at 2 villages: Nady and Sanva in Mounlapamok district 3 months after MDA of praziquantel. A total of 325 villagers participated in this survey. Stool examination by microscopy was conducted by Kato-Katz (KK) method (triplicate) for detecting parasite eggs in the stool samples either on site or at IPL. The results of stool examination were shown in Table 6. In this survey, 69.5% of villagers (226/325) were *O. viverrini* positive, while 0.3% of villager (1/325) was *S. mekongi* positive, including mixed infection. LAMP analysis for detecting *S. mekongi* and *O. viverrini* is now being conducted at Tokyo Medical and Dental University, Japan. A manuscript of these data is in preparation for publication.

Table 6. Results of KK method (Triplicate) in Mounlapamok district, Champasak province in May 2015

Parasites	No.	%
Opisthorchis O. vierrini (Ov)	157	48.3
Hookworm	24	7.4
Ov + Hookworm	53	16.3
Ov + *S. mekongi*	1	0.3
Ov + *T. trichiura*	3	0.9
Ov + Taenia	6	1.8
Ov. + Ascaris	2	0.6
Ov + Hookworm + *E vermicularis*	1	0.3
Ov + Hookworm + *T. trichiura*	2	0.6
Ov + Hookworm + Taenia	1	0.3
Negative	75	23.1
Total	**325**	**100.0**

E. vermicularis: Enterobius vermicularis, T. trichiura: Trichuris trichiura

Opisthorchiasis Survey, Yommalath district, Khammouane province on 16th-26th August 2015

Lao Ministry of Health has been conducting MDA of praziquantel in combination with health education/promotion for villagers in the endemic provinces of Opisthorchiasis for control of the disease. However, infection rate of Opisthorchiasis has been still high in the endemic areas. To find a breakthrough for the control, we attempted to find out positive deviance in the villagers. Positive deviance approach is to identify good practices that are linked to good outcomes, such as preventing malnutrition of children, and infectious diseases.

This survey was conducted at 5 villages (Meuang, Na Phaimai, Nong Ping, Don Phuay, Nagatang) in Yommalath district 8 months after MDA of praziquantel. Yommalath district is known to be one of the high Opisthorchiasis endemic areas in Laos. A pair of 348 mothers and one of their children in the villages participated in this survey (total 696 villagers). Detail questionnaire survey was conducted with mothers, and stool samples were collected from both mothers and one of their children. Consequently, stool samples were collected from 687 villagers. Nine samples were not collected in this survey. Stool examination by microscopy was conducted by Kato-Katz (KK) method (triplicate) for detecting parasite eggs either on site or at IPL. The result of stool examination was shown in Table 7. A manuscript of these data is in preparation for publication.

Table 7. Results of KK method in Yommalath district, Khammouane province in August 2015

Village	No. of examined	*O. viverrini*		Hookworm		*Trichuris trichiura*		Taenia		*Enteroblus vermicularis*		*Paragonimus*		*Fasciola hepatica*		*Ascaris lumbricoides*	
Meuang	258	232	90%	119	46%	38	15%	5	2%	2	1%	1	0%	0	0%	1	0%
Naphaimai	128	113	88%	45	35%	5	4%	3	2%	0	0%	0	0%	2	2%	1	1%
Nongping	92	77	84%	24	26%	5	5%	0	0%	2	2%	0	0%	0	0%	0	0%
Donphuay	83	71	86%	33	40%	9	11%	1	1%	0	0%	0	0%	0	0%	0	0%
Nagatang	126	112	89%	22	17%	5	4%	3	2%	1	1%	0	0%	0	0%	1	1%
Total	687	605	88%	243	35%	62	9%	12	2%	5	1%	1	0%	2	0%	3	0%

Opisthorchiasis Survey, Yommalath district, Khammouane province on 20th-30th September 2015

This survey was conducted at 5 villages (Meuang, Na Phaimai, Nong Ping, Don Phuay, Nagatang) in Yommalath district 9 months after MDA of praziquantel. This was the second survey to find out positive deviance that was associated to prevent *O. viverrini* (*Ov*) infection. We visited 44 households (families) to observe their kitchen, cooking, and washing utensils and interviewed their eating habit as well. Those 44 households were selected based on the results of the first Opisthorchiasis survey in Yommalath district in August 2015. They were 10 *Ov* negative families (both mother -/child -), 34 *Ov* half-positive families (mother + /child -), and 1 *Ov* full-positive family (both mother +/child +). In this survey, we also collected stool samples from all family members and examined by Kato-Katz (KK) method (triplicate) for detecting parasite eggs either on site or at IPL.

We found that *Ov* negative families tended to clean their kitchen and utensils consciously compared to *Ov* positive families. For example, cutting boards at *Ov* negative families were clean, and in fact, such families had more than 1 cutting boards: one for cutting fish or meat and the other for cutting vegetables (Figure 2 & 3). This finding may be a positive deviance for preventing *Ov* infection. The data are now being analyzed at the University of Tokyo.

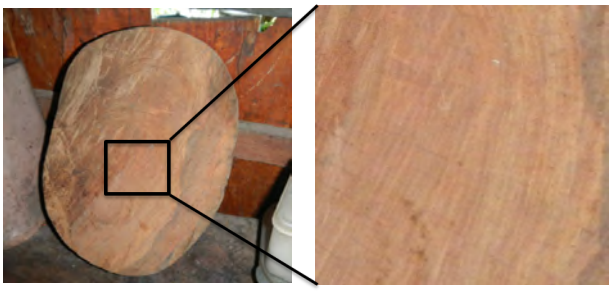

Figure 2. Cutting board in a kitchen at *Ov* negative family. The surface of the board was clean.

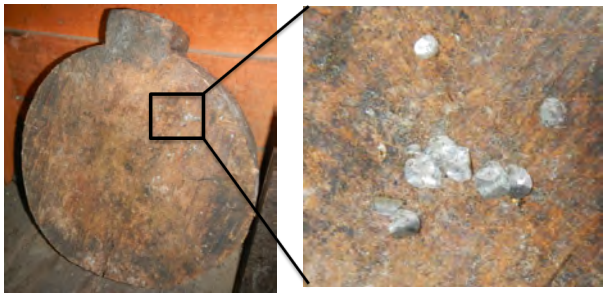

Figure 3. Cutting board in a kitchen at *Ov* positive family. Many fish scales were on the surface of the board.

Discussion of Opisthorchiasis survey in Champasak and Khammouane provinces in 2014 and 2015

Lao Ministry of Health has been conducting MDA of praziquantel and health education/promotion for control of trematode infections, especially Opisthorchiasis in several provinces and Schistosomiasis in Champasak province. However, the infection rate of Opisthorchiasis has still been high in several endemic areas. This can be explained by low coverage of the MDA and re-infection of *O. viverrini* by eating raw or insufficiently cooked freshwater fish that is contaminated with *O. viverrini* metacercariae. Low coverage of praziquantel MDA may be due to its severe side effects, such as, severe epilepsy, rash, nausea, headache, dizziness, abdominal pain, or diarrhea. Thus, there are strict inclusion/exclusion criteria of praziquantel MDA made by Lao Ministry of Health. According to the criteria, people with chronic disease, epilepsy, central nervous system problem, pregnancy and lactating woman will be excluded. This is the limitation of praziquantel MDA. Therefore, health education is crucial to make people aware of the need to stop eating raw or undercooked fish and to use latrine in their villages.

Training courses of parasitic disease diagnosis for medical lab technicians at IPL and provinces

Summary of the training courses at IPL and provinces

The objective of this training course is to increase knowledge and improve skills of effective diagnosis of malaria and other parasitic diseases for medical lab technicians at provincial and district levels in 5 southern provinces (Savannakhet, Saravane, Sekong, Champasak and Attapeu), that are now reported to be high malaria endemic provinces in Laos. Medical lab technicians or staff members from Malaria Station in provincial capital and district, District Hospitals and Health Centers attended the training courses. Target districts were selected based on Annual Parasite Incidence (API: number of malaria case/1,000 population/year) reported by CMPE in 2013. We conducted 3 training courses at IPL and 5 training courses at the provinces. Detail of participants of the training courses is shown in Table 8.

Table 8. Number of participants from the 5 southern provinces

Province	District	API	HC	DH	DM	PM	Total
Savannakhet	Thapangthong	49.6	6	1	1	1	9
	Nong	20.7	8	1	1		10
	Phin	17.5	7	1	1		9
	Sepone	15.8	12	1	1		14
	Vilabury	15.4	8	1	1		10
Saravane	Toumlane	113.1	7	1	1	1	10
	Vapy	68.9	5	1	1		7
	Ta oi	51.4	8	1	1		10
Sekong	Lamarn	22.9	6	1	1	1	9
	Thateng	8.7	8	1	1		10
Champasak	Pathoumphone	53.8	8	1	1	1	11
	Mounlapamok	36.9	6	1	1		8
	Khong	35.0	12	1	1		14
Attapeu	Phouvong	103.4	5	1	1	1	8
	Sanamxay	59.9	8	1	1		10
	Saysetha	31.2	5	1	1		7
Total			**119**	**16**	**16**	**5**	**156**

Training at IPL: 10th-14th, August 2015
Training at IPL: 13th-17th, July 2015
Training at IPL: 22nd-26th, June 2015
Training at Province: October 2015

API: Annual Parasite Incidence (No. of malaria case/1,000 population), HC: Health Center, DH: District Hospital, DM: District Malaria Station, PM: Provincial Malaria Station.

Training at IPL:

- Attapeu (12) on 22nd-26th June 2015
- Sekong (11) and Champasak (15) on 13th-17th July 2015
- Savannakhet (17) and Saravane (14) on 10th-14th August 2015

Training at provinces:

- Savannakhet (35) on 19th-20th October 2015
- Champasak (18) on 22nd-23rd October 2015
- Saravane (13) on 26th-27th October 2015
- Sekong (8) on 28th-29th October 2015
- Attapeu (13) on 31st October 2015

Numbers in parentheses represent number of participants of the training.

Blood sample collection from malaria suspected patients at Hospitals and Health Centers in the 5 southern provinces

We provided filter papers (FTA™ Classic Card, GE Healthcare Life Sciences, Whatman™, UK) for collecting blood samples from malaria suspected patients to the medical lab technicians (participants) of the training courses. The first sample collection was conducted at the 5 southern provinces on October 2015 from the participants who attended our training courses at IPL on June, July and August 2015. A total of 4,441 blood samples were collected from malaria suspected patients (both positive and negative by microscopy or RDT). About 13 % of the samples (587/4,441) were malaria positive by microscopy or RDT (Table 9). In Laos, standard diagnostic method for malaria is microscopy at hospitals level, while RDT is used at community level where microscope is not available.

Malaria DNA analysis is now being conducted using those filter paper blood samples. First, malaria parasite species will be identified by the nested real-time PCR. Second, mutation of drug resistant genes, such as K13, *pfcrt*, will be examined using *P. falciparum* positive samples. Third, population genetic analyses will be conducted using highly polymorphic DNA markers of malaria parasites, such as microsatellite DNA loci to understand transmission dynamics of malaria in Laos (Iwagami et al., 2012 & 2013).

Table 9. Number of filter papers collected from malaria suspected patients in the 5 southern provinces in October 2015

Province	Sampling period	Malaria positive				Malaria Negative	No Data	Total
		Pf+	*Pv*+	*Pf*+ *Pv*+	Unkown species			
Savannakhet	17 Aug – 16 Oct, 2015	20	15	0	24	621	100	780
Saravane	17 Aug – 23 Oct, 2015	10	8	0	23	496	0	537
Sekong	20 July – 27 Oct, 2015	19	37	1	99	771	2	929
Champasak	20 July – 21 Oct, 2015	68	103	5	95	557	20	848
Attapeu	29 June – 30 Oct, 2015	16	33	0	11	1,204	83	1,347
Total		**133**	**196**	**6**	**252**	**3,649**	**205**	**4,441**

RDT: Rapid Diagnostic Test (Malaria Ag *Pf* / *Pv*, Standard Diagnostics, Inc. Republic of Korea)

Partners

- Center of Malariology, Parasitology and Entomology (CMPE), Ministry of Health, Vientiane Capital, Lao PDR
- National Institute of Public Health (NIOPH), Ministry of Health, Vientiane Capital, Lao PDR
- Department of Communicable Diseases Control (DCDC), Ministry of Health, Vientiane Capital, Lao PDR
- Department of Training and Research (DTR), Ministry of Health, Vientiane Capital, Lao PDR
- Department of Hygiene and Health Promotion (DHHP), Ministry of Health, Vientiane Capital, Lao PDR
- National Center for Global Health and Medicine (NCGM), Tokyo, Japan
- Department of Community and Global Health, School of International Health, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan
- Department of Global Health, Graduate School of Health Sciences, University of the Ryukyus, Okinawa, Japan
- Department of Molecular and Cellular Parasitology, Juntendo University School of Medicine, Tokyo, Japan
- Section of Environmental Parasitology, Department of International Health Development, Division of Public Health, Graduate School, Tokyo Medical and Dental University, Tokyo, Japan

Scientific communications

Publication:

Didier Ménard, et al; KARMA Investigators. A Worldwide Map of *Plasmodium falciparum* Artemisinin Resistance. *New England Journal of Medicine*, 2016 (accepted for publication)

Oral presentations:

Moritoshi Iwagami, Phonepadith Khattignavong, Pheovaly Soundala, Lavy Lorphachan, Sengdeuane Keomalaphet, Mixay Phommakhod, Bouasy Hongvanthong, Paul T. Brey, Shigeyuki Kano. Current Malaria Situation in Lao PDR based on DNA analysis. The 9th National Health Research Forum, Vientiane Capital, Lao PDR, October 13th-14th, 2015.

Phonepadith Khattignavong, Enn Sayavong, Pheovaly Soundala, Masami Nakatsu, Satoshi Nakamura, S. Maithaviphet, C. Keomanivong, K. Silavong, B. Phommavongsa, K. Keokenchanh, T. Keobouachanh, H. Keomanila, S. Chanthavongsa, Bouasy Hongvanthong, Moritoshi Iwagami, Shigeyuki Kano, Paul T. Brey. Detection of *Plasmodium falciparum* and *P. vivax* in *Anopheles* mosquitoes in Lao PDR by DNA analysis. The 9th National Health Research Forum, Vientiane Capital, Lao PDR, October 13th-14th, 2015.

Masamine Jimba, Hitomi Araki, Ong I Ken, Moritoshi Iwagami, Bouasy Hongvanthong, Paul T. Brey, Shigeyuki Kano. Positive Deviance Approach for Overcoming NTDs. The 9th National Health Research Forum, Vientiane Capital, Lao PDR, October 13th -14th, 2015

Tiengkham Pongvongsa, Panom Phongmany, Bouasy Hongvanhthong, Daisuke Nonaka, Jun Kobayashi, Moji Kazuhiko, Shigeyuki Kano. Malaria Situation and Challenges in Savannakhet Province. The 9th National Health Research Forum, Vientiane Capital, Lao PDR, October 13th -14th, 2015

Takashi Kumagai, Moritoshi Iwagami, Masafumi Yamabe, Phonepadith Khattignavong, Lavy Lorphacan, Pheovaly Soundala, Bouasy Hongvanthong, Nobuo Ohta, Paul T. Brey, Shigeyuki Kano. The Evaluation of the Molecular Diagnosis using LAMP method against the Parasitic trematoda in Champasak Province, southern Lao PDR. The 9th National Health Research Forum, Vientiane Capital, Lao PDR, October 13th -14th, 2015

Moritoshi Iwagami, Phonepadith Khattignavong, Pheovaly Soundala, Lavy Lorphachan, Sengdeuan Keomalaphet, Mixay Phommakhod, Bouasy Hongvanthong, Paul T. Brey, Shigeyuki Kano. Genetic Epidemiology of Malaria in Lao PDR by SATREPS Project. The 56th Annual Meeting of the Japanese Society of Tropical Medicine. Osaka, Japan, December 4th-6th, 2015

Poster presentation:

Kumagai Takashi et al. *Schistosoma mekongi* and *Opisthorchis viverrini* infection in Khong district in Lao PDR. The 9th Annual Meeting of Helminth Research, Iwate, Japan, July 17th-18th, 2015

Shigeyuki Kano. Development of Innovative Research Technique in Genetic Epidemiology of Malaria and Other Parasitic Diseases in Lao PDR fro Containment of Their Expanding Endemicity. The 30th Annual Meeting of Japan Society of International Health, Kanazawa, Japan, November 21st, 2015

Kei Mikita, Masami Nakatsu, Akihiko Doi, Noppadon Tangpukdee, Srivicha Krudsood, Shigeyuki Kano. Polymorphisms in *Plasmodium falciparum K13*-propeller from Than-Myanmar border in 1996-1997. Joint International Tropical Medicine Meeting 2015, Bangkok, December 2nd-4th, 2015

Moritoshi Iwagami, Phonepadith Khattignavong, Pheovaly Soundala, Lavy Lorphachan, Sengdeuane Keomalaphet, Mixay Phommakhod, Bouasy Hongvanthong, Paul T. Brey, Shigeyuki Kano. Current Malaria Situation in Lao PDR based on DNA analysis by SATREPS Project. Joint International Tropical Medicine Meeting 2015, Bangkok, Thailand, December 2nd-4th, 2015

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They visited Institut Pasteur du Laos!

14 August 2015. Visit of the French Ambassador, H.E. Claudine Ledoux (second from right)

4 August 2015. Visit of the Lao Military Medical Department and Military Institute of Preventative Medicine

13 November 2015. Visit of Mr. Watanabe, President of the "Association Pasteur Japon"

25 February 2015. Opening ceremony of JICA/JST SATREPS Project. From the 6th on the left, Dr. Bouasy (director of CMPE), Prof. Kano (Director of Department of Tropical Medicine of NCGM), Dr. Brey (Director of IPL), H.E. Kishino (Ambassador of Japan), H.E. Dr. Dalaloy (Honorary Chairman of the Board of Directors of IPL, former Minister of Health), Dr. Bounlay (Director of DCDC)

15 January 2015. Visit of D-TRA (Defense Threat Reduction Agency of USA) delegation leaded by Mr. Andrew P. Hollands, Cambodian and Lao PDR country manager (second from left)

4 August 2015. Dr Paul Brey, director of IPL with Ms. Truong Thi Mai, Politburo Member and Party Central Committee's Mass Mobilization Commission of the Socialist Republic of Vietnam (on his left side) and Ms. Vanhpheng Keonakhone, Member of the National Assembly of Lao PDR (on his right side)

2 February 2016. Lao-Luxembourg laboratory PARECID 2 opening ceremony. H.E. Prof. Eksavan Vongvichit, Minister of Health of Lao PDR (on the center), Mr. Claude Jentgen, Charge d'Affaires from Luxembourg Embassy (on his left side) and Prof. Claude Muller, Director of Lao-Lux Lab (on his right side).

Teaching/Training

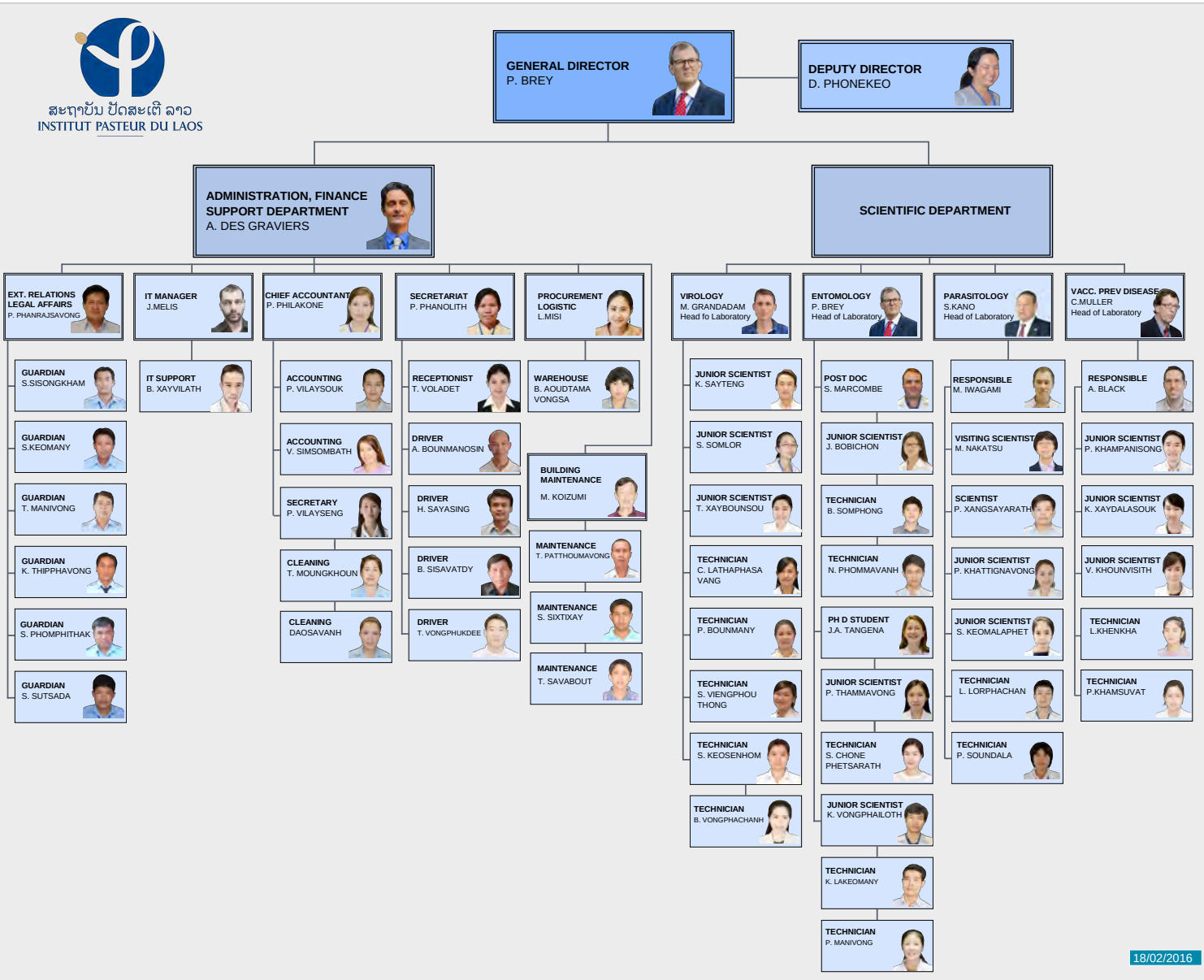
17 December 2015. Diploma received by Mr. La Xayasith after his completion of the entomology training at IPL.

In-house English lessons.

14 August 2015. Training on “Basic Knowledge of Malaria and Other Parasitic Disease Diagnosis” for Provincial Hospitals staff. 69 people were trained during the 3 courses which took place in 2015.

24 August 2015. PACS implementation training.

Main organigram



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