ກະຊວງ ສາທາລະນະສຸກ
Ministry of Health

ສະຖາບັນ ປັດສະເຕີ ລາວ
INSTITUT PASTEUR DU LAOS

Institut Pasteur du Laos

Activities Report 2013

Réseau International des Instituts Pasteur

Summary

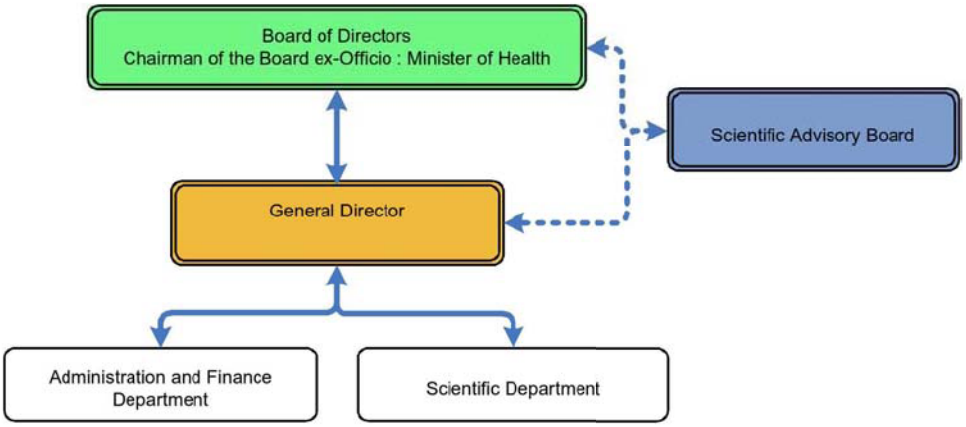
Mandate

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 diagnostic 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

PL 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



Board of directors

IPL is governed by a Board of Directors composed of 3 Lao Members appointed by 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.
Pr. Dr. Bountiem PHISSAMAY, Minister to the Government Office, President of National University Council, Lao PDR.
Mrs. Khampheng PHOLSENA, Minister to the Government Office, President of the National Commission for the Advancement of Women, President of the National Commission for Mothers and Children, 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.

Letter from
Dr. Paul BREY
Director

For many years there was discussion within the Lao Ministry of Health about the possibility to establish an Institut Pasteur in Lao PDR in order to promote research and training and also to provide the Ministry of Health with evidenced-based results to help formulate National health policy. Lao PDR, like Vietnam and Cambodia and China, wanted its own modern research facility to investigate infectious diseases.

In 2003, with the emergence of SARS coronavirus and Avian Influenza H5N1 in Southeast Asia, the former Minister of Health, Dr. Ponmek Dalaloy consulted with the Lao Government and made a formal request to Institut Pasteur in Paris. Dr. Dalaloy stated that Lao PDR was ill equipped and ill-staffed to deal with emerging infectious disease and needed a modern research facility to diagnose and mitigate emerging and re-emerging vial infections. Dr. Dalaloy said he "did not want Lao PDR to be a disseminator of emerging viruses". Dr. Dalaloy and the Lao government renewed their request to establish an Institut Pasteur in Laos (IPL) for several years. After a number of exploratory feasibility studies, Institut Pasteur in Paris agreed to assist the Lao government to establish the Institut Pasteur of Laos as a Lao national institute for research and training on infectious diseases (confer statutes of IP Laos) and on the 16 November 2007 the former Prime Minister, Bouasone Bouphavanh, signed a decree for the creation of Institut Pasteur of Laos.

A year after the Prime Minister's signature the world economy plunged into the most severe economic recession since of the great depression of the 1930's jeopardizing the newly promulgated Institut Pasteur of Laos. However, the team in charge of the fund raising and construction of the Institute, under the guidance of Dr. Ponmek Dalaloy, slowly but surely achieved their objective and the Institut Pasteur of Laos was inaugurated on the first day of the Chinese New Year, 23 January 2012.

The next phase was to establish and equip laboratories within the new institute and hire Lao researchers. During this phase it was decided by the Director of IP Laos and the Board of Director of IPL chaired by Dr. Eksavang Vongvichit, Minister of Health, to create so-called "joint-labs", a novel paradigm, where countries with strong relations to Lao PDR (France, Luxembourg, Japan,…) are willing to support a specific joint-lab by providing a foreign head of lab who is an expert in a specific area of infectious disease research, support Laos scientific staff working in the lab, research costs, as well as contribute to the running costs and administrative support of the institute. Hence, 4 labs were created under this concept:

Lao- French lab for Arbovirology and Emerging viruses
Lao-French Lab for Medical Entomology
Lao -Luxembourg Lab for Vaccine preventable diseases
Lao -Japan Lab for Parasitology

The aim of each lab is to carry out research on specific infectious disease agents affecting the public health of the people of Lao PDR, such as Malaria, Dengue, Chikungunya, Measles, Mumps, Rubella, water and food-borne trematodes etc. in order to provide the Ministry of Health with evidence-based results to contribute and direct National health policy. Furthermore and most importantly, the Lao Ministry of Health has given IPL the challenge to train and mentor a new generation of Lao doctors and scientists who in due time will replace the foreign experts. In addition, IPL has the responsibility to provide assistance to National Centers such as National Center for Laboratory and Epidemiology (NCLE) National Center for Malariology Parasitology and Medical Entomology (CMPE) and the National Center for Animal Health (NCAH) to help them improve their diagnostic capacity and participate in outbreak investigations.

Since opening in January 2012, the Institute has been already actively involved in the surveillance identification and genotyping Dengue and Chikungunya viruses, as well as an outbreak of Mumps. Furthermore, the IPL Parasitology team has refuted through evidence-based results that the claimed presence of Schistosomiasis *(Schistosoma mekongi)* in the tourist city of Vang Vieng (Vientiane Province). Allowing this city to regain its reputation as a major tourist site.

The staff of the Institut Pasteur of Laos , both national and foreign, are here to serve the Lao people though the study of infectious diseases and by training a new generation of researchers to understand infectious disease transmission and to mitigate these threats by informing the Ministry of Health so it can take the appropriate action.

Scientific Activities 2013

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

Since January 2012, the A&EVD laboratory made links with the NCLE to improve surveillance, diagnosis and research on arboviral infections in Lao PDR. Networks were developed by the A&EVD laboratory in order to complement the national surveillance systems. The A&EVD strategy is based on a global approach of arboviral infection. That way, strong internal interactions were put in place with the medical entomology unit. As a complement of the syndromic survey and the existing laboratory surveillance organized in 6 provinces by the NCLE, the A&EVD laboratory set up a hospital network in Vientiane city and different provinces.

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

Scientists:
Malayvanh LAO, MD, Junior Scientist
Sivilay SAYAHEUANG, MD, Junior Scientist (up to September 2013)
Sompavanh SOMLOR, MD, project leader (since November 2013)
Kouxiong SAYTENG, MD, Junior Scientist

Technicians:
Chintana LATHAPHASAVANG, Technician
Phaithong BOUNMANY, Technician
Souksakhone VIENGPHOUTHONG, Technician
Sitsana KEOSENHOM, Lab & Quality Agent

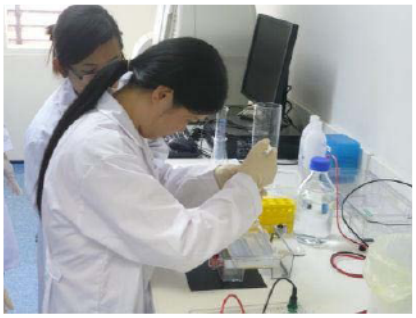
Projects

- Dengue surveillance in Vientiane city
- Transmission cycles of Chikungunya Virus
- Japanese encephalitis and vector virome

Dengue surveillance in Vientiane city

Since 2012, a permanent laboratory surveillance system for dengue has been implemented in Vientiane city by the Institut Pasteur du Laos (IPL) in collaboration with 5 hospitals and the medical centre of the French Embassy.

Coordinator: Malayvanh LAO
Staff: Phaithong BOUNMANY



Laboratory confirmation of suspected cases

Contribution to the global surveillance

For the second consecutive year, the Institut Pasteur du Laos (IPL) actively contributes to improve dengue diagnosis for six clinical facilities (five hospitals and one private medical centre) in Vientiane city. This network allows following a large portion of the population of the city with a major proportion of Laotians representative of the general population in terms of age and sex but also expatriates both residents or tourists. Thus, circulation of dengue can be explored from an "autochthonous" and a regional point of view through imported cases. This system fully complements the provincial survey of the National Centre for Laboratory and Epidemiology (NCLE). Information feedback is a central issue of IPL's network. Priority is given to communication with clinicians in order to provide a direct benefit for the patients' management. A specific diagnosis, based on analysis of patients' clinical and epidemiological data, has been set up to select the most appropriate laboratory tool (s) for samples investigations. Direct (realtime RT-PCR; NS1 antigenemia; viral culture) and indirect methods (rapid test, ELISAs) can be deployed offering a broad range of markers. Weekly reports are provided at different levels, thus data can be integrated to the National surveillance system: (i) a laboratory surveillance report is published weekly by NCLE. This report provides an accurate follow up of the proportion of confirmed cases and a comprehensive overview of the proportions of the dengue serotypes circulating in provinces covered by laboratory surveillance. Figures 1 and 2 are updated in each report. (ii) an independent report, based on syndromic recording of the suspected cases is updated daily by NCLE. At this stage, a cross analysis is still missing especially for the numeration of confirmed deadly cases.

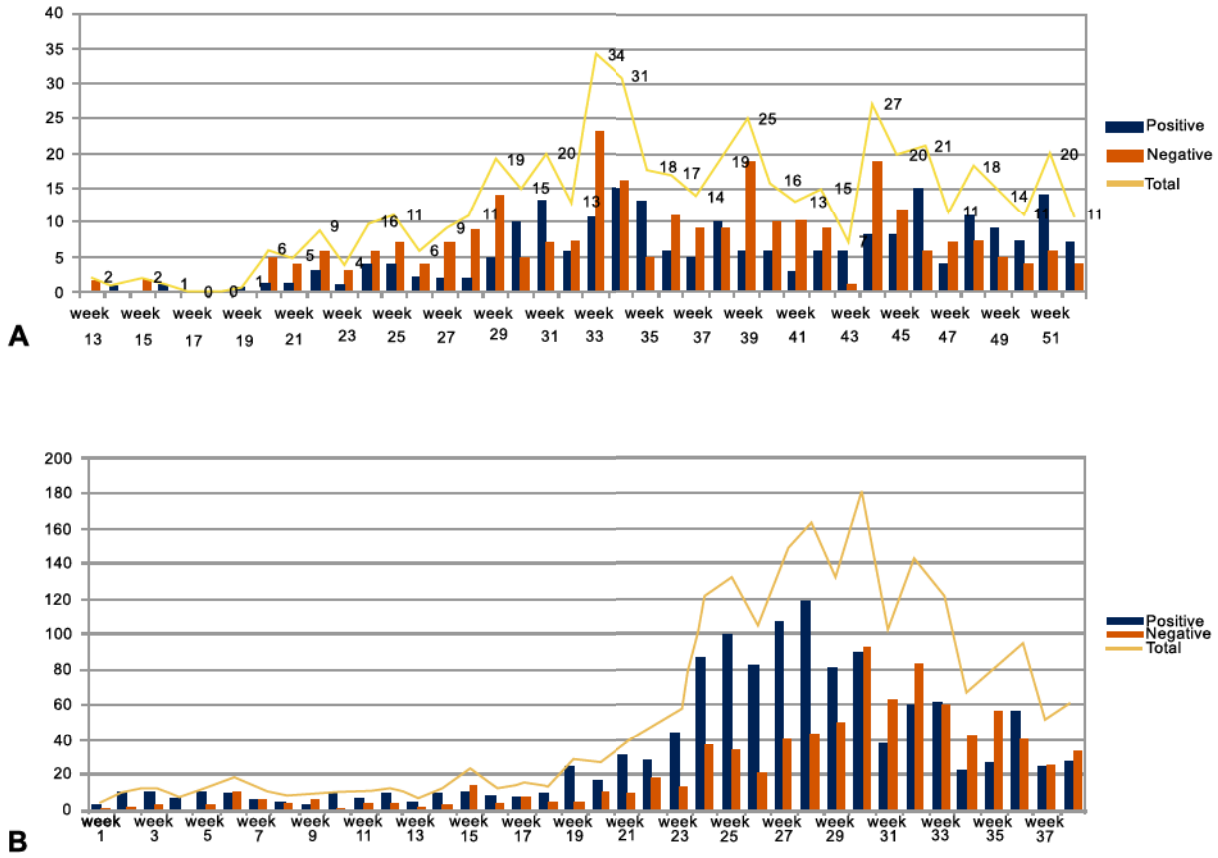


Figure 1: Dengue laboratory surveillance in Vientiane Capital.
Surveillance held by Institut Pasteur du Laos in 2012 (A) and in 2013 (B).
Yellow curves represent the total number of dengue suspected cases investigated.

Severe forms / deadly cases

Since January 2013, 19 deadly cases were recorded by the IPL hospitals network and investigated dengue markers. For 15 of these cases, death could be confirmed to have occurred in a dengue infection context. To our knowledge, no alternative etiology could be evoked for the four remaining cases raising questions of the origin of these fatal issues and of the possible co- circulation of other(s), pathogen(s). Dengue typing was performed on all cases who had positive RT-PCR (n=9). All cases were linked to dengue 3 (Table I). This could be expected due the predominance of this serotype. Complete sequences are ongoing to further characterize the viral strains.

Table I: Classification of dengue deadly cases recorded by the IPL network

GROUPS	CRITERIA	Nb of cases/ serotype
CONFIRMED	Direct diagnosis (RT-PCR; NS1; V.C)	9/DENV3=4, DENV1=1 ,DENV ON STUDY=4
PROBABLE	Presence of IgM and/or IgG	7/NA
NOT CONFIRMED	No marker	4/NA
	TOTAL	20

V.C.: viral culture; NA: Not applicable

Data collection

Confirmed cases can be mapped by exploiting the IPL questionnaire. However only home addresses are available; no GPS positioning is possible yet. Personal information is available for most cases investigated. Furthermore, possible imported cases could also be deduced from data collected. Vector control campaigns were started too late to envisage targeted actions based on personal data.

Serotypes and distribution

Dengue typing is performed on all positive samples detected by mean of a pan-dengue real-time RT-PCR. Figure 1 show the distribution recorded in Vientiane Capital since April 2012. In 2013, typing could be performed until the end of March. The huge increasing of positive samples can no longer allow maintaining a systematic investigation. Thus, only a subset of samples was analyzed weekly to keep an eye on dengue serotypes. Up until now, nearly 80% of the virus typed belonged to serotype 3. The remaining 20% are dengue 1 or undetermined samples (low viral load) as a consequence of a lower sensitivity of the typing method compared to the screening technique.

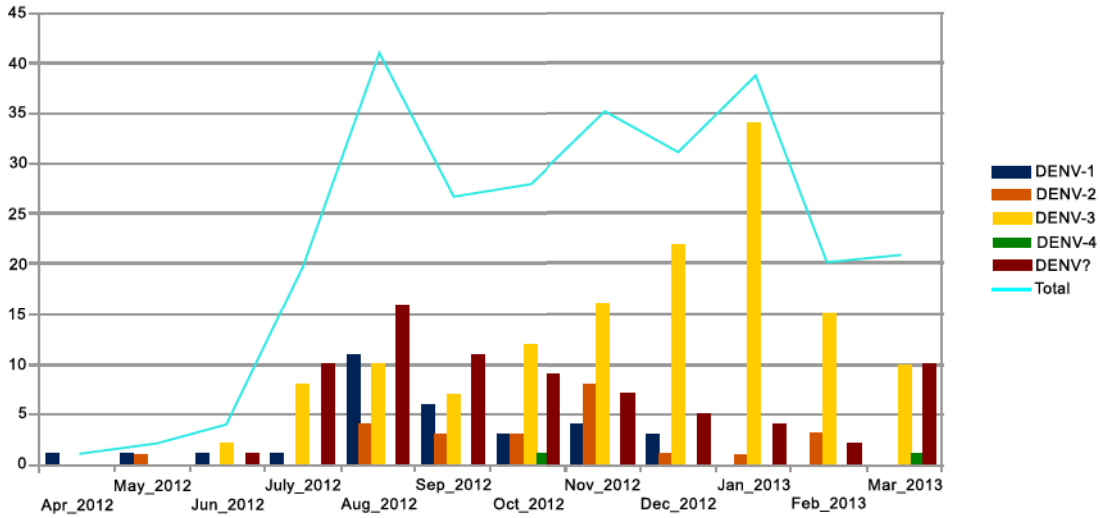


Figure 2: Follow up of dengue serotypes in Vientiane city.

Dengue virus serotypes

Dengue typing is performed routinely on all positive samples detected by mean of a pan-dengue real-time RT-PCR. This approached allowed detecting the emergence and spreading of dengue serotype 3 in June 2012. Dengue 3 serotype can be considered to be still predominant at least in Vientiane city. Sequence data evidenced the presence of two different genotypes: (i) genotype II has been detected since June 2012 in the capital but also in some provinces (Vientiane province; Luang Prabang; Champassak and Atteupeu). Most of the strains typed fall in this genotype. This group of stains could be linked to dengue isolates from Myanmar. (ii) In late December 2012, and second genotype has been detected in Vientiane city (2 strains). The origin of these strains has not yet been fully determined and need more extensive sequence data. At last, the distribution of the two genotypes in 2013 remains to be investigated.

Feed back to clinicians

Samples collection is organized two times per week by the IPL staff. Results are communicated to clinician by phone and a specific report is edited the each patient and send to the clinician. However, in case of emergency, samples can be picked up in the hospitals by IPL or bring to the institute for rapid analysis. Clinicians can directly contact IPL staff to discuss specific cases that may require a rapid investigation. IPL can mobilize staff to perform analyses during the week end.

Providing data for vector control activities

As shown in figure 1, the number of cases dramatically increased since August. Thus, targeted vector control became useless to fight efficiently against viral transmission. However, the weekly report clearly mentioned the situation in each district of the capital with reference to the number of cases confirmed in districts and villages.

Transmission cycles of Chikungunya Virus

Coordinator:
Sivilay SAYAHEUANG,
(up to September 2013)
Sompavanh SOMLOR,
(since November 2013)
Staff:
Chintana LATHAPHASAVANG

In late July 2012, Chikungunya virus infections were confirmed by the NCLE. The medical entomology and A&EVD units actively participated to the investigation of this first outbreak by performing an active entomologic investigation in the emergence foci and characterizing the viral isolates at the genetic level. The work of the Institut Pasteur's units continued after the official statement of the end of the epidemic in September 2012.

Prevalence study in two districts of Champasak province

A prevalence study has been organized in collaboration with the Institut de la francophonie pour la médecine tropicale in the two districts struck by the 2012 outbreak. A total of 568 volunteers were sampled in 26 villages. Anti-chikungunya virus antibody detected by ELISA revealed a predominance of recent exposure profiles. Among these, 6% displayed only anti-CHIKv IgM compatible with the stage of infection. The reinvestigation of this group's samples allowed detecting two viremic sera confirming the active circulation of the virus. Seroprevalence levels recorded in the village ranged from 33 to 94% without evidence of a clear geographical gradient. This heterogeneity could be connected at least in part to the rural nature of this region.

Active surveillance in Southern provinces of Lao PDR

To follow the spreading of Chikungunya virus in Lao PDR an active surveillance network has been set up with the provincial hospital surrounding Champasak provinces (i.e. Sekon; Salavanh and Atteupeu). A total of 398 blood samples from patients presenting symptoms compatible with an acute phase of Chikungunya virus infection were investigated by RT-PCR. Out of 387 patients from Champasakprovince, 43 were found positive. Of 20 declared patients from the 3 other provinces (Sekon, n=17; Salavanh, n=2 and Atteupeu, n=1) only one patient from Sekon province was found positive.

Japanese encephalitis and vector virome

Japanese encephalitis is a viral mosquito-borne infection transmitted to humans in rural environments mainly by *Culex tritaeniorhynchus*. Japanese encephalitis virus (JEv; *Flaviviridae*, genus *flavivirus)* is maintained in the environment through an enzootic cycle where migratory birds and pigs play the role of amplifying hosts. JEv is considered as the major cause of viral encephalitis in young children in Southeast Asia with a high impact in terms of morbidity and mortality. Lao PDR is a rural country where large areas of rice paddies offer optimal conditions for JEv maintenance. However, JEv epidemiology is poorly documented in Laos. For instance only one genome sequence has been published so far.

Coordinator: Kouxoing XAYTENG
Staff: Souksakhone VENGPHOUTHONG

Localization of transmission foci

To document the genetic diversity of Japanese encephalitis virus in Lao PDR and investigate the role of others flaviviruses in the etiology of viral encephalitis in Lao PDR.
Serum samples from pigs, collected in slaughter houses in Vientiane province are investigated for the presence of anti-Japanese encephalitis virus antibodies. Information on breeding sites of the positive pigs are collected and used to map the possible transmission area of the virus and organize entomologic investigation. Mosquito collected in different regions of Laos are also systematically investigated for the presence of JEv genome. Samples from human suspected cases recorded in the hospital network of Vientiane city are investigated for confirmation.

An in-house competitive ELISA test has been set up for the serological investigation of pigs' samples. Over 211 pigs sera investigated, 21% were found positive. Mapping of the breeding sites allowed identifying 7 potential sites of active or recent transmission of Japanese encephalitis virus.

Study of arthropods' virome

As a complementary activity of different projects (i.e. PTR 408; ACIP 2012-16), the investigations of arthropods' virome captures during different campaigns were systematically investigated by broad range RT-PCR methods.
Chikungunya virus could be detected and isolated from Aedes aegypti caught in epidemic foci in Champassak province.
Dengue could be isolated from conventional but also from unconventional mosquito species. Natural dengue and Chikungunya co-infection could be evidenced in Aedes aegypti.
Alphavirus and flavivirus sequences could be detected by RT-PCR in mosquitoes collected in remote areas of Lao PDR.
Presence of phlebovirus sequences could be evidenced in different species of sandflies from rural areas of Laos.
Sequencing and isolation assays are ongoing.

Funds and collaborations

Funds

Institut Pasteur du Laos, International division, Institut Pasteur, Paris: ACIP 2011-16; PTR 408.

Donations

- Division International
- Institut Pasteur Paris
- ST Bank
- IRVTUS3N Singapore
- AusAID/WHO
- Embassy of the Feeral Republic of Germany
- Phongsavanh Bank
- Exotissimo travel Co.,ltd
- Lao Brewery Co., LTD
- Phu Bai Mining limited
- Banque Franco -Lao ltd

Collaborations

Institut Pasteur du Laos
Lao-Luxemburg Laboratory (A. Black)
Medical entomology unit (P.T. Brey; K. Vongpailoth; S. Marcombe).
Institut Pasteur de Paris (V. Caro ; L. Diancourt; J-M Thiberge).
Institut Pasteur's international network
Institut Pasteur du Cambodge
Institut Pasteur de Madagascar
Institut Pasteur du Sénégal.

Scientific communications

Oral communications

M. Grandadam; M. Lao; K. Vongpailoth and P.T. Brey. Institut français du Laos, Vientiane, Lao PDR. Etat de la situation sur la dengue et le Chikungunya au Laos. September 18th 2013. Invited conference.

M. Grandadam. Practical step by step approaches for improving provincial laboratories. Regional workshop: Improving laboratory services for communicable diseases in the Mekong sub region. Vientiane, Lao PDR. October 1st 2013.

M.Lao,P.Bounmany,B.Oudavong,K.Choumlivong,M.Thongsana, C.Vanhlasy,K. Boudsada, J-M.Hospied, M.Grandadam, P.Brey. Detection and typing of dengue strains in Vientiane Capital, Lao PDR. Third International Conference on Dengue and Dengue Haemorrhagic Fever, 2013. Bangkok, October 21-23rd, 2013.

Scientific publications

Articles:

Dupont-Rouzeyrol M, Caro V, Guillaumot L, Vazeille M, D'Ortenzio E, Thiberge JM, Baroux N, Gourinat AC, Grandadam M, Failloux AB. Chikungunya virus and the mosquito vector Aedes aegypti in New Caledonia (South Pacific Region). Vector Borne Zoonotic Dis. 2012 Dec;12 (12):1036-41

Contribution to books:

[West Nile virus and phylogenetic analysis]. M. Grandadam; C. Renaudat. 2013. In : Le virus du Nil Occidental. Ed. D. J. Bicout. Editions QUAE. Chapter 2 :25-42.

Rift Valley fever virus. Manual of security sensitive microbes & toxins. Dongyou Liu Ed. Taylor and Francis Group. Boca Raton.

Diagnosis of West Nile virus infection]. M. Grandadam; C. Renaudat. 2013. In : Le virus du Nil Occidental. Ed. D. J. Bicout. Editions QUAE. Chapter 6 :105-117.

Education activities



Training and workshops to which A&EVDunit staff contributed

Names	Subject	Organized by	Date	Type
Marc GRANDADAM	Infectious agents and tumors	Institut Pasteur / Fondation Mérieux / IFMT	March 2013 (1 day)	Theory/practical session
Marc GRANDADAM / Malayvanh LAO	ELISA	Institut Pasteur du Laos	June 2013 (1 week)	Theory/practical session
Marc GRANDADAM / Malayvanh LAO	ELISA	Medical academy of the Lao Army	June 2013 (2 days)	Theory/practical session
Marc GRANDADAM	Regional workshop on chikungunya virus	Institut Pasteur du Laos	7-11 October 2013	Theory/practical session
Malayvanh LAO	Conference on Chikungunya virus infection	Institut Pasteur du Laos / IFMT	9 October 2013	Theory

Training of students

Names	Degree	Institution by	Time period	Subject
Athinna NISAVANH	IVolunteerMaster 1 graduated in 2012)	IUniversité Pierre & Marie Curie, Paris VI. Paris, France	March-May 2013	Sanflies and phebovirus infections in Lao PDR
Julie BOBICHON	Master 1	Université J. Fournier. Grenoble, France	April-June 2013	Characterization of anti-flavivirus antibodiesusing native and viruslike particles as antigens
Mingyuang LI	PhD	university of Hong-Kong - Li Ka Shing Faculty of Medicine	April – August 2013	Interaction of KDLER pathway with Dengue virus cellular life cycle
Sompavanh SOMLOR	Master 2	IInstitut de la francophonie pour la medicine tropicale	March to September 2013	Seroprevalence of chikungunya virus in Champasak province

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

The main objective of our lab is to study the biology, 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

Scientists:
Sébastien MARCOMBE, Ph D

Junior Scientist:
Khamsing VONGPHAILOTH, MD
Phoutmany THAMMAVONG, MD
Outhévanh KOUNNAVONGSA, MD

PhD Student
Julie Anne TANGENA, MSc

Projects

- Risk Of Vector-Borne Diseases In Relation To Rubber Plantations In Lao PDR
- Malaria vectors in Lao People's Democratic Republic and Thailand and capacity building in medical entomology (MALVEC)
- Species composition of mosquito fauna from riverside rock pools in the Nakai Nam Theun National Protected Area (NNT NPA)

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

ECOMORE project: ECOnomic development, ecosystem MOdifications,and emerging infectious diseases Risk Evaluation

Coordinator: Dr. Paul BREY/ Julie-Anne TANGENA

Members of staff:
Phoutmany THAMMAVONG
Honglakhone XAIYASING

A

A. Rubber tree with collecting cup

B

B. Rubberplantation

C

C. Cutting of rubbertree for tapping

Background

China is the second largest global economic power and bordering countries are benefitting from this increased wealth. In 2009 alone 341 billion US dollars' worth of products were imported from neighbouring countries into China (IMF, 2010). One of the products in high demand in China is rubber. Rubber is used for many products including conveyer belts and many adhesives, but mostly for car tyres. In China alone the number of cars has grown 20 fold in the pastdecade with 18.5 million cars sold in 2011 (Branigan, 2012). With only 3% of the 1.3 billion Chinese population owning a car at the moment (Madslien, 2012), the car industries arenot expected to slow in their growth any time soon. During 2012 consumption of rubber increased by 4% with Asia consuming the biggest proportion of natural rubber(Sumernet, 2009). It has encouraged countries to invest inrubber plantations in Asia where 93% of all natural rubberis produced. In the last few decades the number and area of rubber plantations have increased rapidly. Rubber plantations are now found largely in Thailand, Indonesia and Malaysia, together accounting for 72% of all natural rubber production(ANRPC, 2010). 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 30o 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. See figure 1. Every worker is able to tap between 300-450 trees per night equivalent to 1ha of rubber plantation. Lao PDR has seen a high increase in rubber plantations table 1, where relatively low number of plantations are present compared to neighbouring countries (Sumernet, 2009; Hurni, 2008; Li and Fox, 2012). This is a new kind of mass farming not seen in Lao before. After 2015 the area of rubber plantations will continue growing with currently 342,400ha of land designated for rubber plantations (NAFRI, 2011). There are two different kinds of rubber plantations in Lao PDR; small scale Lao owned rubber plantations and industrial scale plantations.

Year	Mature rubber plantation in Lao PDR
2010	900
2011	6,900
2014	28,802
2015	147,500

Table 1: Total hectares of mature rubber plantations in Lao PDR (NAFRI, 2011)

Vector-borne diseases can increase or decrease due to changing land use (de Castro et al., 1999; Yasuoka and Levins, 2007) [59, 60]. As mentioned before in some areas with the massive clearing of the rain forest the typical habitat for An. dirus has decreased and malaria declined (Do Manh et al., 2010). However tree crop plantations can provide the preferred habitat again with canopy cover and ample human hosts. Rubber is known to be a significant site for malaria transmission (Singhasivanon et al., 1999; Yasuoka and Levins, 2007). In an area of Thailand it was estimated that 25.5% of all malaria cases were caused by work on commercial plantations, including fruit, rubber and teak plantations (Singhasivanon et al., 1999). Many thousands of people are employed in the commercial forest industry, most of whom spend considerable time in and close to these forests when An. dirus is biting. Rubber plantation tappers are expected to be most at risk as they work outdoors at night when the malaria vector is most active. In 2012 malaria incidence in the south of Lao PDR was three times as high as 2011 (WHO, 2013). This increase in malaria incidence is not well understood but could be related to the increase in rubber plantations, with malaria risk three times higher for people working in forested areas then people living in residential villages (Erhart et al., 2005).

The influence of rubber plantations in Lao PDR on the spread of dengue and chikungunya remains unclear. Aedes albopictus and Ae. aegypti are known to easily adapt to new environments. In rubber plantations discarded containers used for collecting latex are common and may provide ideal breeding sites for these mosquitoes. Furthermore depending on the vector species able to invade the rubber plantations and the proximity to forests, emerging infectious diseases could be of interest.

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 (NAFRI, 2011). 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 back home. Even more worrying is the possibility to introduce a drug resistant strain, like ACT resistance, in an area where the vectors are already present by either returning home with a resistant strain or transporting the resistant strain to the place of work.

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.

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

Methods

Field sites
Our first aim was to find a suitable area to conduct our study. We needed to establish three areas of around 4km2 where the following four habitats were present; immature rubber plantation, mature rubber plantation, village and forest. Especially finding a mature rubber plantation, a plantation which has been tapped for at least one year, was difficult. Most plantations were still young and not ready for tapping for another two years. We managed to find the three areas within Luang Prabang province, in bordering Nan and Xieng Ngeun distict. The villages consist of Lao Loum and a mix of minorities including Khamou, Yao and Hmong.

Mosquito ecology in rubber plantation
To understand the ecology of the mosquitoes in the rubber plantations, we are comparing the mosquito number and diversity of the plantation with the forest and village throughout the rainy season. We started with the Human Landing Catches method, which entails the use of human participants sitting in the different habitats collecting all mosquitoes landing on their legs throughout a time period. However as a dengue epidemic occurred during our collection period, we decided to shift to double bednet collections to ensure participants are not exposed to mosquitoes. The double netting method entails the use of a small net covering the participant and a larger net covering the smaller net with a gap of 30cm at the bottom. Mosquitoes are attracted to the presence of the participant and fly towards them, mostly flying low to the ground. These mosquitoes encounter the smaller bednet and try to gain access by flying up. They are now in between the two bednets and will have difficulty escaping. See figure 2. Every hour the participant within the net collects all the mosquitoes in between the two bednets and puts them in one collecting cup for each hour. Hereby we collect data on the density and diversity of mosquitoes every hour, both day and night, every month during the rainy season. With this data we can analyse the behaviour of mosquito species attracted to humans and their abundance in the different habitats. All methods were approved by the Lao Ethics committee.

Figure 2: images of double netting method (A) double netting method including a bamboo construction to hang bednets from and keep participants comfortable (B) for illustration of the double netting method, the pink bednet is the small net which reaches the floor, covered by a blue bigger net which is about 30cm from the ground (C) a participant is collecting mosquitoes between the two nets

Resistance study

For possible implementation of vector control methods in the future it is of vital importance to understand the resistance status of the important vector mosquitoes. As Anopheles species were too low for good resistance analysis and malaria cases were not found in our study area we focussed on the dengue vector Aedes albopictus. These mosquitoes were collected from the secondary forest in Village No 7 by villagers using the double netting method. Two participants collected mosquitoes in the forest for three consecutive days. These mosquitoes were exposed to insecticides in WHO tubes for one hour in our field laboratory. Knock down and mortality was noted. The insecticides used were DDT (4%), permethrin (0.75%) and bendiocarb (0.1%).

Figure 3: Field laboratory

Larval survey

To understand where mosquitoes breed in the rubber plantations, monthly surveys were done in both mature and immature rubber plantations. Random collecting cups were checked and all possible breeding sites were analysed, including areas around the houses of the rubber tappers within the plantations.

Trapping comparison

For further entomological surveys in rural areas of Laos it is important to understand the benefits and costs of different surveying methods, especially when human participants are used. Many different trapping methods are possible for surveys,including the CDC light trap, the BG sentinel trap and the Suna trap. We are comparing the different traps with the double netting method to understand how efficient and reliable the different traps are in comparison. Not enough data has been collected for analysis in this annual report yet.

Results JUNE - AUGUST 2013

Mosquito ecology in rubber plantation

A total of 10,332 female mosquitoes have been collected and identified during the last three months, consisting of fifteen whole days (0:00-0:00) with three participants in each habitat. Unfortunately not all data from the first month have been identified yet, due to the late delivery of the microscopes nor all tubes from September due to ill health of one of our members of staff. This data is just a first indication.

Currently we have collected 113 different species of which 32 *Aedes* species and 22 *Anopheles* species. The possible malaria vectors collected are at least: *An. maculatus*, *An. minimus*, An. baimaii (*dirus s.l*), An. barbirostris and *An. pampanai*. The total number of mosquitoes collected in the forest is much higher than for the other habitats, with immature rubber > mature rubber > Village. See table 2. This seems to be mostly due to the high numbers of Aedes and Armigeres species in the forest. It seems that for the dengue and chikungunya, exposure risk is highest in secondary forest, followed by high risk in both types of rubber plantations and low risk in the village. For Japanese encephalitis only the possible vector *Culex vishnui* was found. The exposure risk seems to be highest in the village, with many rice fields in close proximity. Furthermore both secondary forest and immature rubber plantations show high numbers.

Although mosquito numbers are high in the secondary forest, this does not entail *Anopheles* species are the highest there. Both immature rubber plantations and villages show highest number of possible malaria vectors. An average of 2 possible malaria vectors are collected from each participant every night for both village and immature rubber plantation. The most common vector was An. maculatus, followed by An. barbirostris and *An. minimus.*

Table 2: Total number of mosquitoes in each habitat, including the possible vector species and species which showed high numbers throughout the collection period

Total no. mosquito	Secondary forest	Mature rubber	Immature rubber	Village
All species	5849	1606	2392	1022
Aedes species	2122	931	1106	142
Aedes albopictus	1555	628	680	55
Heizmania chengi	573	100	237	3
Heizmania mattinglyi	846	275	550	10
Armigeres kesseli	1146	21	9	38
Armigeres flavus	180	45	40	7
Culex vishnui	254	48	126	365
Culex whitei	159	23	77	145
Anopheles species	88	28	90	166
Possible vector of malaria	57	21	79	80
An. maculatus	24	16	66	33
An. minimus	4	1	1	26
An. baimaii	2	1	7	0
An. barbirostris	27	3	4	19
An. Pampanai	0	0	1	2

Figure 2: shows the total number of *Aed. albopictus* for all habitats combined, through time. The number of albopictus increase after 5:00 in the morning, then becomes constant for a few hours. After 12:00 it starts increasing again until it reaches its peak between 15:00 and 17:00, after which the numbers start decreasing again. It will be interesting to understand why the number of mosquitoes start increasing after 12:00. The temperature and humidity data will be included in the near future to understand if it explains the increases and decreases seen in the figure.

Figure 1: Number of Aedes albopictus through time, combining data of months, locations and habitats

No possible malaria vectors were collected during the day.

The most noticeable conclusion from *Anopheles* data is that the activity of all possible malaria vectors is between 18:00 at night and 6:00 in the morning. See figure 4. High number of *An. maculatus* were collected during the first half of the night with a peak at 20:00.

Resistance study

In total 12 exposures were conducted over three days in three different months. Of these exposures three were control, three Bendiocarb, two DDT and four permethrin exposures. All control exposure tubes consisted of at least 12 female Aedes albopictus mosquitoes. No control mosquito died during the 24hrs after exposure. All insecticide exposed mosquitoes died 24hrs after exposure. See table 3. High knock down was seen

	Total no. mosquitoes exposed	Knock down after 15 min.	Knock down after 30 min.	Knock down after 45 min.	Knock down after 60 min.	Dead after 24hrs.
Bendiocarb	56	12	47	56	56	56
DDT	26	0	2	17	22	26
Permetrhin	76	56	75	76	76	76

Table 3: WHO tube test results for bendiocarb, DDT and permethrin

Larval survey

We did not manage to find any larvae in the collecting cups of rubber plantations on the trees as all plantation workers were actively tapping rubber throughout the rainy season. The cups containing water were not left long enough for mosquito larvae to develop with every tree in the three plantations being tapped at least every three days. However we did find a few larvae in an unused rubber collection cup found on the floor. We found most Aedes larvae in man-made constructions, including gates, fences and other bamboo constructions, and in garbage left by tappers and villagers. Furthermore mosquito larvae were found in natural slow moving streams and marshes at the bottom of rubber plantations. See figure 5.

Figure 5: From left to right showing places where larvae were found; pieces of cut bamboo, a half cut bottle, an unused rubber collecting cup, the marshes at the bottom of the rubber plantations in Huay Hoi village

Important Meetings

We have had several important meetings with key people from the village, district and province throughout this year. Every activity organized in our study area was always first discussed and approved on country level, provincial level, district level and village level. On provincial and district level both the Ministry of Agriculture and Ministry of Health were involved. All village meetings were documented, including number of participants, names, sex and age. Some villages have more meetings documented then others, due to an extra meeting held to find new participants as a high number of participants travelled to work abroad. Furthermore for our trapping comparison experiment in village No.7 we needed an extra village meeting.

Village meetings No. 7: 02/06, 07/07, 07/08, 09/09
Village meetings Silalek: 17/06, 14/07
Village meetings Huay Hoi: 04/06, 07/07, 04/09

Future Activies

We will continue collecting mosquitoes both day and night in each habitat during the dry season with monthly intervals. Furthermore we will conduct molecular analysis to further understand which species in our habitats are a vector for dengue. As malaria cases were not found in our study area during the rainy season, molecular analysis for malaria will not be conducted. Not only Aedes albopictus will be tested, but other possible dengue vectors will also be analyzed including Armigeres species. Additionally Rural Appraisals will be organized with the villagers to gain more insight into their behaviour towards and knowledge on vector-borne diseases. Using the data from these results we will try to identify the possible risk for vector-borne diseases in the rubber plantation and, if possible, test some vector control methods.

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

Coordinator: Sebastien Marcombe

Members of staff:
Outhevanh Kounnavongsa

Anopheles dirus female taking a blood meal

Meeting with the villagers
before night mosquito collection

House selected for the collection

In Lao PDR, a recent national survey on the distribution of malaria showed that 65% of the population was still living in transmission areas. 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 Lao PDR, a recent national survey on the distribution of malaria showed that 65% of the population was still living in transmission areas. 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. 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. 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. It is possible that the use of agricultural insecticides may be at the origin of the selection of these resistances nd so constituting a danger for the implementation of effective vector control strategies. a 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
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.

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

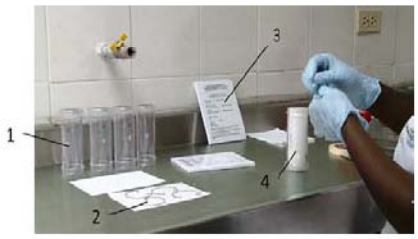
- Set up a comprehensive map representing the "hot spots" for malaria transmission in Lao PDR and Thailand (border area)
- Generate an Insecticide Resistance database in the main malaria vectors
- Address the dynamics and gene flows between malaria vectors populations - Guide public health authorities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

Partners

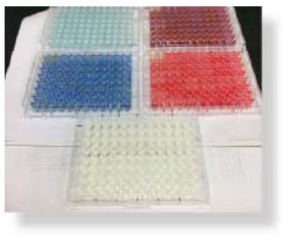
- National Center of Malariology, Parasitology and Entomology (CMPE), Vientiane, Lao PDR
- Institut de Recherche pour le Développement (IRD) IRD-MIVEGEC, IRD UMR-MD3, Bangkok Thailand
- Kasetsart University, Department of Entomology, Bangkok, Thailand
- Institut de Médecine Tropicale d'Anvers (IMTA), 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



WHO test on adult mosquitoes



Biochemical assays

Species composition of mosquito fauna from riverside rock pools in the Nakai Nam Theun National Protected Area (NNT NPA)

Nakai district, Khammoune province, Lao P.D.R
and preliminary arboviral detection studies.

Coordinator: Dr. Paul BREY

Members of staff:
Khamsing VONGPHAYLOTH

Background

Arthropod-borne viruses (arboviruses) are emerging and re-emerging viruses that are important causative agents of human disease in many areas of the world, especially in tropical and subtropical areas. The most important arbovirus families are *Flaviviridae* (including dengue and Japanese encephalitis viruses) and *Togaviridae* (including Chikugunya virus). Most arboviruses circulate among wild animal populations; they are transmitted between vertebrate hosts by blood feeding arthropod vectors, such as mosquitoes, sandflies and ticks. Transmission can also be vertical (the passage of the virus from an infected female mosquito vector to both male and female offspring) or horizontal (acquired from one infected vertebrate host during the vector blood feeding stage) (Weaver and Reisen 2010). Anthropo-zoonotic arboviruses can spill over into humans upon entry into localities where zoonotic cycles are occurring (forest, caves, etc). This can be followed by sustained human-to-human transmission via relay vectors when the humans return to rural or urban environments. Chikungunya virus, for example was originally maintained in sylvatic vertebrate hosts and mosquito vectors then spilled-over to humans, who brought the virus back into rural and urban environments where sustained human-to-human transmission occurred via *Aedes aegypti* and/or *Aedes albopictus* serving as effective urban/rural vectors.

Nearly a half a century ago, Halstead and Udomsakdi (1966) reported on anthropo-zoonotic cycles of Chikungunya in humans, primates and other vertebrate hosts in Southeast Asia. Primates, horses, pigs and water buffalo were found to have titers of Chikungunya neutralizing antibodies. However, these zoonotic cycles were never studied further. More recently, during an arbovirus sero-surveillance study in resettlement populations of the Nam Theun 2 hydroelectric project (2007 & 2010), neutralizing antibodies for Semliki-forest group of alphaviruses including Chikungunya were detected in 3.7 -15.9% of the population depending on the village (Marc Grandadam, Personal Communication). Interestingly, few *Aedes albopictus* and no *Aedes aegypti* mosquitoes were found in the resettlement villages suggesting that the Chikungunya transmission was taking place elsewhere. Discussions with resettlement villager populations indicated that these populations were actively involved in hunting, fishing and gathering on the Nakai Plateau but also throughout the Nam Theun watershed including the Nakai Nam Theun National Protected Area (NNT NPA) covering a total area of 423 000 hectares. This area is considered as a biodiversity hotspot in Southeast Asia. Recent data gathered from several investigations in the area, have identified more than 400 species of birds, 92 species of mammals, 29 species of reptiles, and 25 species of amphibians (Watershed Management and Protection Authority [WMPA] magazine). These results prompted us to investigate a putative anthropo-zoonotic cycle for Chikungunya, as well as other arboviruses vertically transmitted in putative mosquito vectors.

Preliminary mosquito surveys on Nam Noy River in the Nakai Nam Theun National Protected Area (NNT NPA) during the dry

and rainy seasons of 2011, at location latitude: N17°76733 and longitude: E105°38140 (Figure1), revealed two putative mosquito vectors for anthropo-zoonotic arboviral circulation (*Aedes. elsiae* and *Aedes. macfarlanie*). These species have a highly specific breeding habitat (rock pools) that support mosquito breeding throughout the year regardless of rainy season or dry season. Putative vertebrate hosts for Chikungunya and other arboviruses come into contact with these species as they go to drink from the rivers. In 2012 and 2013 we undertook extensive mosquito surveys to further identify putative sylvatic Chikungunya /arbovirus vector species. The mosquito material was then processed for RT-PCR for Chikungunya and other arboviruses and preliminary results are given.

Objective

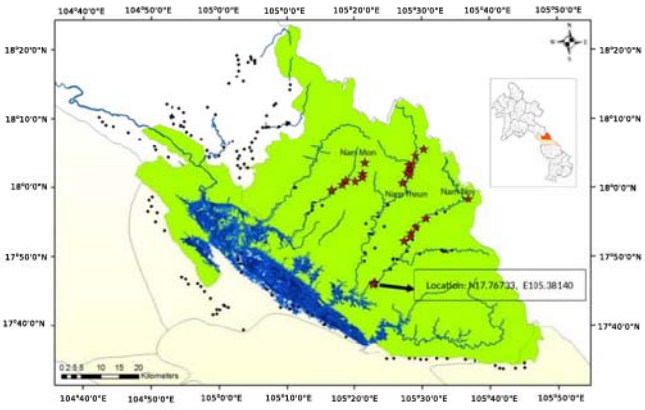
To evaluate the risk of arboviral transmission in the forest of Nakai Nam Theun National Protected Area (NNT NPA) area by:

1. Continuing mosquito larval sampling in many locations of the area.
2. Describing vector species composition and distribution from larval sampling.
3. Molecular investigation of arboviral vertical transmission

Methodology

Study sites: the mosquito larval surveys were carried out in the forest in Nakai Nam Theun National Protected Area (NNT NPA) area, where the sylvatic and vertical transmission cycles of arboviruses are hypothesized to exist, located in Nakai district, Khammoune province, in the central part of Lao PDR. Three main rivers within the Nakai Nam Theun National Protected Area (NNT NPA) were selected for mosquito larval sampling (Figure 1). Larval habitats, especially the rock pools, alongside the river were investigated.

Figure 1: Map showing locations of larval mosquito sampling in three main rivers in Nakai Nam Theun National Protected Area (NNT NPA) area. Red stars were the sites that



Mosquito larvae were sampled.

Mosquito sampling and identification: Mosquito immature stages were collected extensively from all larval habitats found in the field, especially rock pools alongside of three main rivers (namely Nam Noy, Nam Theun and Nam Mon), using a standard 250 ml larval dippers and pipettes on 7 different occasions from January 2012 to March 2013.

Larval collections from Rock Pools in the Nakai Nam Theun National Protected Area (NNT NPA)

Larvae and pupae were transferred to labeled bottles (location, date), then transported to the IP Laos Nakai Field Laboratory to rear and identify. In the lab, the larvae and pupae from each bottle were counted by classifying into three groups of development stages, stage 1-2; stage 3-4; and pupae then transferred to a labeled cup (the same as bottle label) covered with permeable gauze netting to allow air circulation but preventing the escape of adults. Larvae and pupae were reared to adults. It should be noted that viral RNA extraction is much easier from adult mosquitoes than larvae that contain high amounts of fat body, that compromises efficient viral RNA extraction. Adult mosquitoes were aspirated and transferred to another labeled cup and killed by freezing at -30 °C, 5 min then identified morphologically using keys to the adult mosquitoes of Thailand (Rattanarithikul et al. 2005, Rattanarithikul et al. 2006, Rattanarithikul et al. 2010) and Vietnam (Stojanovich and Scott 1965). Each adult mosquito was dissected in two parts. Abdomen, legs, wings from mosquitoes sorted by sex, species and location were grouped in pools that did not exceed 10 specimens. The head – thorax segments of each specimen were stored in single tubes. All material was stored at -80 °C for further viral detection and isolation.

Nucleic acid extraction and RT-PCR

Detection of viral sequences was first conducted on pools. Each pool containing abdomen, legs and wings was mixed with PBS and ceramic beads and crushed using an automatic tissue homogenizer. One half of the supernatant was used for RNA extraction. Purified RNA was used as matrix for molecular screening using pan-flavivirus and pan-alphavirus conventional RT-PCR. Amplicons were detected by agarose gel electrophoresis. Head – thorax segments from positive specimens were submitted to the same procedure to determine

the specific infection rates and for isolation in cell culture. Viral identification was attemped by retesting positive samples by specific real time RT-PCRs as follows: (i) pan-flavivirus: dengue (universal and serotype specific), Japanese encephalitis and (ii) pan-alphavirus: Chikungunya virus.

Results

Larval collection

Over the course of sampling between 2012 and early 2013, a total of 24,221 larvae and pupae were collected from three main rivers of investigation. 12,828 (52.96%) and 8,509 (35.13%) larvae were collected from Nam Noy and Nam Theun River respectively. 13,198 and 2,031 were the 2nd and 3rd stages and pupae respectively (Table1).

Table 1: Number of mosquito in each stages of development collected from three main rivers.

River	Development stages							
	L1-2	%	L2-3	%	Pupae	%	Total	%
Nam Mon	672	7.47	1,842	13.96	370	18.22	2,884	11.91
Nam Noy	5,522	61.41	6,583	49.88	723	35.60	12,828	52.96
Nam Theun	2,798	31.12	4,773	36.16	938	46.18	8,509	35.13
Total	*8,992*	100	13,198	100	2,031	100	24,221	100

Mosquito species of adults emerging from larval collection

A total of 4,082 adults were emerged, of which 2,178 (53.36%) and 1,904 (46.64%) were females and males respectively. A total 21 mosquito species were found, of which 8 species were belong to 6 genera of mosquito in the tribe *Aedini* composing of - (1) genus *Collessius* : Ae. (*Collessius*) elsiae and *Ae.* (*Collessius*) *macfarlanie*; (2) genus *Fredwardsius* : *Ae.* (*Fredwardsius*) *vittatus*; (3) genus *Gilesius*: Ae. (*Gilesius*) *pulchriventer*; (4) genus *Hulecoeteomyia*: *Ae.* (*Hulecoeteomyia*) *chrysolineata* and *Ae.* (*Hulecoeteomyia*) *saxicola*; (5) genus *Scutomyia*: *Ae.* (*Scutomyia*) *albolineata* ; (6) genus *Stegomyia*: *Ae.* (*Stegomyia*) *albopicta*. 3 species were belong to genus *Anopheles* (Meigen, 1818) of which 2 species were in subgenus Cellia: *An.* (*Cellia*) maculatus subgroup and *An.* (*Cellia*) *pseudowillmori*. 7 species were in the tribe *Culicini* comprising one subgenera of *Lutzia* and 4 subgenera of *Culex* (Table2).

Ae.(*Col.*) *elsiae* and *Ae.*(*Col.*) *macfarlanie* were the most abundant species found in three main rivers of our investigating sites. *Ae.*(*Col.*) elsiae was the most frequent species in Nam Noy and Nam Theun, represented (1,982/4,082) 48.55% of total mosquito emergence. *Culex* in the subgenus *Culiciomyia* was the most abundant among *Culex* species, represented (568/4,082) 13.91% (Table2).

Table 2: Mosquito species composition and abundance of adult emerged from larval collection in three main rivers.

Mosquito species	River			Total
	Nam Mon	Nam Noy	Nam Theun	
1. Ae. (Scutomyia) albolineata (Theobald, 1904)	1	0	0	1
2. Ae. (Stegomyia) albopicta (Skuse, 1895)	3	38	0	41
3. Ae. (Hulecoeteomyia) chrysolineata (Theobald, 1907)	5	0	21	26
4. Ae. (Collessius) elsiae (Barraud, 1923)	29	493	1,460	1,982
5. Ae. (Collessius) macfarlanei (Edwards, 1914)	195	446	349	990
6. Ae. (Gilesius) pulchriventer (Giles, 1901)	1	0	27	28
7. Ae. (Hulecoeteomyia) saxicola (Edwards, 1922)	1	9	23	33
8. Ae. spp.	3	0	0	3
9. Ae. (Fredwardsius) vittatus (Bigot, 1861)	0	35	0	35
10. An. (Cellia) maculatus subgroup	7	1	8	16
11. An. (Cellia) pseudowillmori	0	4	0	4
12. An. spp.	2	4	1	7
13. Cx. (Culiciomyia) viridiventer (Giles, 1901)	98	470	0	568
14. Cx. (Lophoceraomyia) Fraudatrix Group (Edwards, 1934; Barraud, 1934)	0	18	2	20
15. Cx. (Lophoceraomyia) Mammilifer Group (Edwards, 1932) and Wilfredi Group (Sirivanakarn, 1977)	16	0	0	16
16. Cx. (Culex) mimeticus complexe	1	0	1	2
17. Cx. (Culex) mimulus complex	8	124	50	182
18. Cx. (Oculeomyia) sinensis	0	12	1	13
19. Cx. spp.	35	10	13	58
20. Lt. (Metalutzia) vorax (Edwards, 1921)	21	29	3	53
21. Ur. (Uranotaenia) macfarlanei (Edwards, 1914)	0	4	0	4
Total	426	1,697	1,959	4,082

Preliminary Arbovirus detection

A total of 53 different mosquitoes species were collected during 2011 and 2012 campaigns mostly on the rivers and to a lesser extend in the villages of the Nakai plateau. A total of 4299 specimens divided in 840 pools were obtained in 2011. Of the 576 pools analyzed at present, 30 specimens were found positive by pan-alpha virus (n=13) or pan-flavi (n=17) RT-PCR (Table 3). In 2012, 3306 specimens were obtained. Of the 571 pools, 528 were analyzed by the pan-alphavirus RT-PCR with one positive (Table 3). None of the 60 pools analyzed at present by the pan-flavivirus RT-PCR was found positive. Among the samples analyzed, 15 different species were found positive for at least one of the two viral genus screened. The pan-flavivirus positive specimens collected in the villages were reanalyzed by specific real-time PCR for dengue and Japanese encephalitis. Two specimens were found positive for dengue 3 and the virus could be isolated from theses specimens (Table 3). These preliminary results demonstrate the large diversity of mosquito species harboring arboviral sequences. Isolation assays and sequencing of pan-alphavirus and pan-flavivirus are ongoing on all positive samples.

	2011		2012	
Genus	Species	Virus identification	Species	Virus identification
Aedes spp	macfarlanei (1A ♀)	NI	Elsiae (1A)†♂	NI
	aegypti (2F ♂♀)	NI	AO	NAY
Anopheles spp	notanandai (1A♀)	NI	AO	NAY
	nigerrimus (1F)	1 DEN3 (i)	AO	NAY
	spp (1F ♂)	NI	AO	NAY
Armigeres spp	subalbatus (2A ♂♀,3F 3♀)	1 DEN3 (i)	AO	NAY
	kuchigensis (2A ♂♀)	NI	AO	NAY
Culex spp	tritaeniorhyncus (2A ♂♀,3F 3♀)	NI	AO	NAY
	vishnui (2A♀, 2F♀)	NI	AO	NAY
	fuscocephala (1A; 3F†2♂1♀)	NI	AO	NAY
	quinquefasciatus (1F♀)	NI	AO	NAY
	whitmorei (1A ♀)	NI	AO	NAY
	sinensis (1F♀)	NI	AO	NAY
	mimulus cx (1A ♂)	NI	AO	NAY
Total	30 positifs (13 A ; 17F)		1 positif (1 A)	

F: pan-flavivirus positive; A: pan-alphavirus positive; NI: not identified; (i) virus isolated; AO: analyses ongoing; NAY: not available yet.

Discussion and perspective

Our surveys showed that 24,221mosquito larvae and pupae representing 21 mosquito species of total 4,082 adult mosquito emergences. Ae.(Col.) elsiae and Ae.(Col.) macfarlanie were the two predominant species representing in the three main rivers of our investigating sites and this two species can be found throughout the year. This is the first study on mosquito fauna and their arboviral vector status in Nakai Nam Theun National Protected Area (NNT NPA). Pan-Alpha, Pan-Flavi samples will be cultured on C6 36 and Vero cells to isolate viruses and subsequently sequenced them in the CIBU at IP Paris.

This project was funding by PTR 408 from IP Paris

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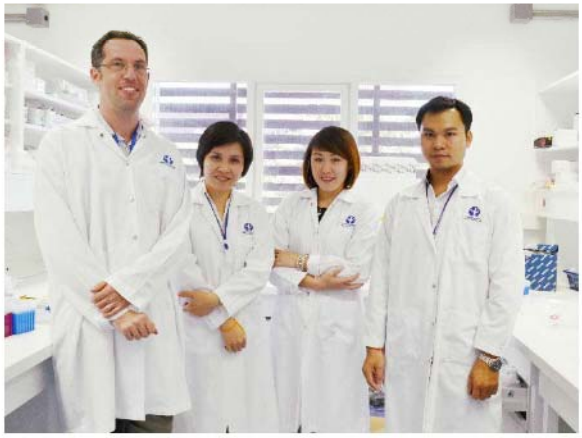
Vaccine Preventable Diseases Laboratory

Lao-Lux joint Lab

The LaoLux Laboratory 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



Head of Laboratory: Professor Claude MULLER

Scientists:
Antony Black, PhD Scientist and Responsible of the Lab
Phonethipsavanh NOUANTHONG, PhD

Junior scientists:
Chanthasone SOUVANNASO, MD
Keooudomphone VILIVONG, MD

Projects

- Mumps seroprevalence and molecular epidemiology
- Hepatitis B and C virus in Lao blood donors
- Hepatitis B and C in Lao healthcare workers
- Measles rubella campaign
- Influenza A Virus and other avian and swine pathogens
- Immune response to measles virus containing vaccine and wildtype measles virus infection: their differential long-term effects with emphasis on the B cell repertoire
- Respiratory infections in 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 CRP-Santé.
- 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 and collaborating scientists from Institute of Immunology, Luxembourg:

Dr. Judith Hübschen.
Dr. Nina Lütteke.
Dr. Axel Dubois.
Dr. Chantal Snoeck.

Students trained in 2013:

Dr. Naphavanh Nanthavong, Institut de la Francophonie pour la Medecine Tropicale

Financial support
The laboratory is funded by a grant from the Government of the Grand Duchy of Luxembourg and is operated by the Institute of Immunology of CRP Santé in Luxembourg

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LABORATOIRE IMMUNOLOGIE

Mumps seroprevalence and molecular epidemiology

Background

Mumps is a vaccine preventable disease caused by mumps virus (family: Paramyxoviridae, genus: Rubulavirus) with a subclinical course in up to one third of all cases. Common manifestations in all patients include parotitis and respiratory symptoms and orchitis in postpubertal males. Laboratory diagnosis is usually done by detecting specific IgM antibodies or mumps virus RNA. Based on 316 nucleotides covering the small hydrophobic (SH) gene region, 12 different genotypes of mumps virus have been proposed with genotypes C, F, G and H predominating in Asia . In Lao People's Democratic Republic (PDR), mumps is not a notifiable disease and mumps vaccine is currently not included in the routine childhood immunization schedule. While no data exist for previous years, a total of 54 cases were reported to WHO in 2011 . Studies investigating the seroprevalence of mumps-specific IgG antibodies are lacking for Lao PDR and thus the public health burden of mumps in this country is currently unknown.

Activities

The Lao Lux laboratory, in collaboration with NCLE, carries out passive surveillance of mumps cases within Lao PDR. Seroprevalence data are also determined on a country-wide scale.

Throat swabs and sera were collected from clusters and sporadic cases between 2011 and 2013. For RNA positive samples detected by reverse transcriptase PCR, 316 nucleotides covering the small hydrophobic gene region were used for phylogenetic analyses with MEGA 4 software. Furthermore, sera collected from healthy infants and pupils between 9 months and 19 years and from pregnant women between 16 and 46 years were investigated for mumps-specific IgG antibodies using commercially available ELISA kits.

Our data indicate widespread wild-type mumps infections within Lao PDR and the presence of genotypes G and J. These studies are the basis for a first assessment of the public health benefit of including mumps in the EPI programme.

Prospective

These data were presented as an oral presentation at the National Health Research Forum in Vientiane, October 2013. A manuscript of these data has been submitted. Laolux laboratory continues active mumps surveillance in collaboration with NCLE.

Partners

- Department of Pathology, University of Health Sciences, Vientiane, Lao PDR.
- Luangprabang Provincial Hospital, Luangprabang, Lao PDR.
- Department of Pathology, Faculty of Medicine, Chiang Mai University, Thailand.
- WHO Lao Country Office, Vientiane, Lao PDR.
- National Center for Laboratory and Epidemiology (NCLE), Vientiane, Lao PDR.
- Public Health Laboratory Services Branch, Centre for Health Protection, Department of Health, Hong Kong SAR, China.

Women and children attending a community study information meeting in a school in Huaphan.

Hepatitis B and C virus in Lao blood donors

Background

Hepatitis B virus (HBV) is highly endemic in the Lao People's Democratic Republic. In collaboration with the Lao Red Cross, the Institute of Immunology, Luxembourg has shown that approximately 45.5% of Lao blood donors are positive for at least one hepatitis B virus serum marker; anti-HBs, anti-HBc or HBsAg, suggesting that almost half have been previously exposed to HBV. 9.6% of donors were screened out of the blood donation process, due to HBsAg positivity, indicating a high prevalence of chronic infection.

Furthermore, a large variety of subgenotypes (B1, B2, B4, C1, C5, I1, I2) are present in HBsAg positive donors with subgenotypes B4 and C1 dominating. Mixed genotype infections are also common and found in up to 6% of HBsAg positive donors, with approximately 65% of these infections having recent recombinations within the S-gene alone. These mixed and recombinant infections are highly variable but have not yet led to new dominant strains within the studied population. In more than 30% of donors with recombinant strains, the most closely related known virus was found in the same donor, suggesting recent recombination events. Importantly, two new subgenotypes (I1 and I2) have been identified and characterised, belonging to a recently defined genotype I and representing up to 5% of HBV infections. Molecular analyses suggested that this genotype developed outside Southeast Asia in the distant past by a series of recombinations. Importantly, 3.9% of blood donations were HBsAg negative but DNA positive (occult infected) and potentially infective for HBV. This has implications for the HBV screening of Lao blood donations, which are currently tested only for HBsAg but not DNA.

Other previous work from the Institute of Immunology in Luxembourg, in collaboration with the Lao Red Cross, examined the molecular epidemiology of hepatitis C virus (HCV) from anti-HCV-positive first-time blood donors. Out of 105 samples, forty-five were positive for HCV (42.9%); two belonged to subtype 1b (2/45, 4.4%) and all others to genotype 6 (43/45, 95.6%), including subtypes 6b, 6h, 6k, 6l, 6n and 6q. Three groups of sequences were not clearly attributable to any genotype 6 subtype, two of which may be regarded as candidates for new subtypes of genotype 6. Two samples were mixed infected with different subtypes or clusters of genotype 6 viruses.

Prospective

The LaoLux laboratory continues to work in close collaboration with the Lao Red Cross to further investigate the prevalence and molecular characteristics of hepatitis infections in Lao blood donors. Current investigations include nationwide surveys of the prevalence of occult HBV infections, the incidence of hepatitis D co-infection with HBV and the infectivity of HBV occult infected blood. These studies are an important contribution to the safety of blood recipients and the epidemiology of hepatitis B and C in the young Lao population.

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.

Hepatitis B and C in Lao healthcare workers

Background

Healthcare workers (HCW) are particularly at risk of HBV and HCV infection via bodily fluids due to percutaneous and mucocutaneous exposure. HBV is the most easily transmitted blood borne pathogen and an unvaccinated HCW has the risk of transmission from HBsAg positive needle stick injury of between 6-30%. The WHO estimate that every year in the Western Pacific Region B, which includes Lao PDR, 269,000 and 953,000 HCW are exposed to at least one percutaneous injury with a sharp contaminated with HCV and HBV, respectively. Approximately 41% and 36% of HCV and HBV infections of HCW are attributable to contaminated sharps in Western Pacific region B.

The Advisory Committee on Immunization Practices recommends vaccination of HCW in the United States with several vaccines including 3 doses of HBV. However, the vaccination or infectious status of HCW is often unknown. In Lao PDR, there is no nationwide vaccination schedule for HCW. There are no published data on the added risk of HCW to become infected by vaccine preventable diseases in Laos. Furthermore, lack of resources prevents adequate vaccination coverage and pre and post vaccination testing among HCW.

Activities

The LaoLux laboratory is investigating the HCV and HBV-specific serology in HCW in Vientiane capital and some provincial hospitals in Lao PDR. The prevalence of anti-HBc, anti-HBs, anti-HCV and HBsAg in HCW in different departments and settings with high risk (e.g. infectious disease units, dialysis units, surgery, obstetrics/gynaecology) will be determined. This will provide the basis for a public health strategy to better protect HCW against these and other infections.

801 healthcare workers have been recruited to date from hospitals within Vientiane Capital. Recruitment of participants from provincial hospitals is ongoing.

Prospective

Healthcare worker recruitment continues in Huaphan and Boulhikhamxay provinces. Seroprevalence for other important infectious diseases will also be determined. Laboratory results obtained by the LaoLuxLab will be communicated back to the hospital/health care station/medical staff and the Lao Ministry of Health after completion of the analysis.

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.

Enrolment of participants for healthcare worker study

Measles rubella campaign

Background

Measles is a highly contagious disease affecting more than 20 million people each year. It was estimated that in 2011 about 158 000 deaths were related to measles virus infection. The majority of these deaths (> 95%) occur in resource poor countries with suboptimal health systems and are due to complications such as encephalitis, severe diarrhoea or pneumonia. Vaccination with one dose of measles containing vaccine has been carried out for nearly 30 years in Lao PDR. The estimated routine measles vaccine coverage rates were 52% in 2008; far below the global average of 83% and far below the 95% coverage required to interrupt measles virus transmission. Figures from the Lao Ministry of Health suggest that the vaccination coverage in 2011 was still below 70%.

Infection with rubella virus normally leads to the development of a maculopapular rash with low fever and few complications. However, infection during pregnancy may cause the death of the foetus or severe congenital diseases (congenital rubella syndrome, CRS) in the infant. These congenital infections can affect virtually all organs, but ear, heart and eye defects are the most common manifestations. Rubella vaccines were included for the first time national immunization in the campaign in 2011/2012 in Lao PDR.

Not much is known about the measles and rubella seroprevalence in Lao PDR. In particular, there are no age stratified measles and rubella seroprevalence among different age groups and between the geographical regions of Lao PDR.

As the WHO Western Pacific Region has committed itself to eliminate measles in the region, a population immunity of 95% needs to be achieved. To substantially improve the population immunity to measles in Laos, a supplementary immunization activity (SIA) targeting about 3 million people between 9 months and 19 years of age was carried out at the end of 2011 using combined measles and rubella vaccine.

Activities

To assess the seroprevalence in the different target age groups of the SIA and in a cohort of pregnant women, LaoLux Lab collected serum and oral fluid samples from over 2000 donors between 9 months and 19 years and from pregnant women above the age of 19 years from different geographical regions in Laos prior to the SIA. In order to assess seroconversion following SIA, a second sample from the same people was obtained about 3 months after vaccination.

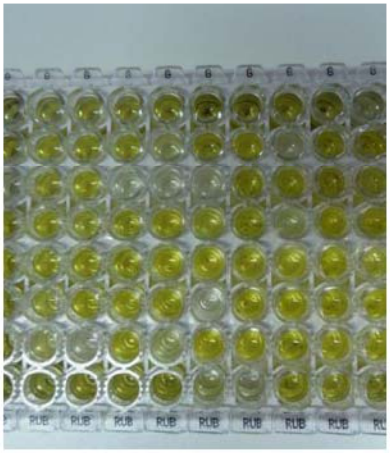
A substantial proportion of the sampled population, including pregnant women, were susceptible to infection prior to the SIA.

Prospective

Paired post-vaccination samples will be tested for seroconversion. The success of the SIA will be estimated on the basis of the second sample and potential problems related to the campaign will be identified. These data will also provide valuable information on the vaccine efficiency in different age groups and geographical regions and on the rubella virus circulation and the risk of CRS in Lao PDR. The results of the study will prove useful to decide on future strategies for both measles and rubella control in Lao PDR.

Partners

- Vientiane central hospitals; Mitthapab, Settathirath and Childrens.
- Vientiane district hospitals; Sykhod, Chantabouly, Sysatanak, Xaysetha.
- Luangprabang Provincial Hospital, Luangprabang, Lao PDR.
- Department of Pathology, University of Health Sciences, Vientiane, Lao PDR.
- Department of Pathology, Faculty of Medicine, Chiang Mai University, Thailand.
- WHO Lao Country Office, Vientiane, Lao PDR.
- National Center for Laboratory and Epidemiology, Vientiane,



Influenza A Virus and other avian and swine pathogens

Background

As in other countries in the region, Lao farmers practice mixed-poultry backyard farming where ducks, geese, chicken and other birds are traditionally reared together. These domestic birds mix with wild waterfowl that are the main reservoir of all subtypes of influenza viruses. This has been the breeding ground for several recently emerging influenza viruses including H5N1 in Hong Kong and elsewhere in Southeast Asia. Thus active surveillance for low and high pathogenic avian influenza in backyard poultry, domestic and wild waterfowl is an important activity both for public health and animal husbandry.

Furthermore, a number of other avian pathogens including Newcastle Disease Virus (NDV), Infectious Bronchitis Virus (IBV), Infectious Bursal Disease Virus, Chicken Anemia Virus (CAV), Infectious Laryngotracheitis Virus and others are of great economic importance and may cause heavy losses to an emerging poultry industry. Therefore it is important to understand which pathogens circulate in poultry and to investigate their prevalence, the genetic variants, the epidemiology, the burden of disease and so on.

Activities

Live bird markets host a wide variety of bird species in close contacts with each other, providing optimal conditions for virus spread over small and long distances, transmission between avian species and potential transmission of zoonotic diseases to humans such as avian influenza viruses. Therefore, swab samples will be collected from live bird markets as a representative of a small region and starting an active surveillance program. Poultry and swine samples will also be collected from mixed farms and swine sera collected from slaughter houses. Samples will be used to investigate the prevalence of influenza A virus and antibodies. Samples from poultry will be used to test for other avian viruses like Infectious bronchitis virus, Newcastle disease virus and Chicken anemia virus. Subsequently PCR will be used to detect the presence of the different viruses. Sera will be analysed by virus neutralization assays using a panel of diverse human and avian influenza viruses for presence of neutralizing antibodies. Phylogenetic analyses will be used to investigate the genotypes, the origin of the virus and routes of transmission between the different hosts.

Activities

Further sample collection and data analysis are ongoing.

Partners

- 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.

Immune response to measles virus containing vaccine and wildtype measles virus infection: their differential long-term effects with emphasis on the B cell repertoire.

Background

The aim of this project is to assess the use of the lymphocyte repertoire as a tool to distinguish between an immunity induced by the measles vaccine or by a wild-type measles virus infection. The measles virus is serologically monotypic and such a distinction is so far not possible. Using deep sequencing technology, we will compare the "signature" of various wildtype and vaccine strains on the lymphocytes repertoire and determine whether particular CDR3s can be used to draw retrospective conclusions about the virus strain responsible for the immunity.

Activities

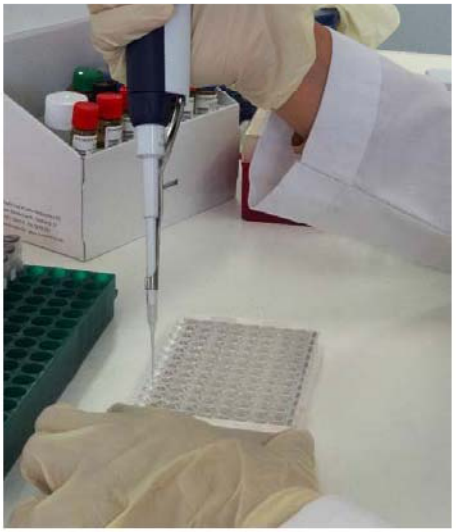
In November 2012, the anti-measles immunity of 200 medical students from Vientiane Medical School was assessed using a commercially available IgG ELISA kit. Out of the 8 negative individuals, 7 accepted to enrol in our vaccination follow up study that took place from December 2012 to January 2013. Blood was taken at different time points after vaccination with a monovalent measles vaccine. mRNA from PBMCs was extracted at the Institut Pasteur du Laos. In addition, 25 cord blood samples have been collected in collaboration with the Mahosot hospital in January 2013.

Prospective

The samples will now be further processed in Luxembourg.

Partners

- Mother and Child Hospital, Vientiane, Lao PDR.
- Medical school, Vientiane, Lao PDR.
- Department of Pathology, University of Health Sciences, Vientiane, Lao PDR.
- Department of Pathology, Faculty of Medicine, Chiang Mai University, Thailand.



Respiratory infections in Lao PDR

Background

Although there are few data concerning viral or bacterial respiratory infections in Lao PDR, these are likely to represent a great burden on the health system. In particular, little is known about the epidemiology of seasonal and pandemic influenza A virus in the country. Systematic serological cohort studies before (stored sera) and after the emergence of pandemic H1N1 (2009) virus are warranted, especially as Lao PDR borders on China which is considered an important hub for influenza virus. Mixed backyard farming of chicken, ducks and swine, all highly susceptible to similar influenza A strains, represent a major risk for the development of reassortant viruses with unpredictable properties.

The Hong Kong influenza pandemic scare of 1997, zoonotic H5N1, the SARS outbreak in Guangdong Province and the swine influenza epidemic 2009 are recent examples of new emerging respiratory viruses that represent major threats to public health and global economy. Although industrialized countries maintain surveillance and early warning systems, control efforts in many DCs including Laos are not always sustained. An important activity of this project consists also to support laboratories to improve their surveillance capacity.

Activities

We will investigate the relative risk of cohorts that have household or professional contacts with pigs, ducks and chicken in comparison with individuals that do not have such contacts. Also, the spontaneous development of drug resistant viruses will be monitored. This may provide a unique opportunity to investigate cross-neutralization between virus strains from different host species (human, avian, swine). Neutralization data paired with full genome analysis will help to better understand opportunities for cross-protection.

Sera from people with close contact to swine (slaughterhouse workers, swine farmers, backyard farmers) and sera from the general population were collected in 2011 in Lao PDR. Samples were analysed by virus neutralization assays for detection of antibodies directed against different Influenza A subtypes. A high prevalence of antibodies against pandemic H1N1 2009 was found among swine contacts. Further local virus strains will be investigated.

In a separate study, 309 nasal swabs from humans with acute respiratory tract infections were collected at Luang Prabang hospital by Ms Valy Phongsavath. In 2011 Ms Phongsavath visited the department of immunology for training on molecular diagnostic with these samples. A high percentage of different respiratory viruses was found.

Prospective

Sample collection and data analysis are ongoing.

Partners

- 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.
- Luangprabang Provincial Hospital, Luangprabang, Lao PDR.

Parasitology

Lao-Japan joint Lab

Aims of the 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**

Scientists:
Satoshi NAKAMURA, Ph D, Visiting Scientist
Masami NAKATSU, Ph D, Visiting Scientist

Junior scientists:
Phonepadith KHATTIGNAVONG, MD
Eng SAYAVONG, MD

Technicians:
Pheovaly SOUNDALA
Lavy LORPHACHAN

Projects

- Distribution of *Plasmodium falciparum* and *Plasmodium vivax* in *Anopheles* mosquitoes in Lao PDR
- Investigation on *Schistosoma Mekongi* in northern Laos

Distribution of Plasmodium falciparum and Plasmodium vivax in Anopheles mosquitoes in Lao PDR

Coordinator:
Dr. Phonepadith KHATTIGNAVONG
Members of staff:
Dr. Lavy LORPHACHAN
Pheovaly SOUNDALA

Summary

Advanced routine PCR system that detects *Plasmodium falciparum* and *P. vivax* parasites in anopheline mosquitoes was established at Institut Pasteur du Laos for the first time in Lao PDR. A total of 38 parasites positive anopheline mosquitoes were detected by this detection method from 1515 samples of the mosquitoes collected by National Centre of Malariology, Parasitology, and Entomology (CMPE) from the Provinces of Phonsaly, Bokeo, Luang Phabang, Vientiane, Bolykhamxay, Saravane, Champasak, and Attapeu in June, 2012. These species were known malaria vectors, such as *An. minimus* (26), *An. maculatus* (8), and *An. jeyporiensis* (3) and *An. philippinensis*(1). As for the parasites, 34 mosquito cases of *Plasmodium falciparum* and 5 cases of *P. vivax* infections were confirmed independently; however, only one case of mixed infection was among the sample. Domestic distribution of the *Plasmodium falciparum* malaria positive mosquitoes was the highest in a village in Saravane of the eight Provinces sampled.

Introduction

Malaria is still a very important infectious disease in Lao PDR, and the elimination of the disease is regarded as high priority and the duty of the Lao PDR government (National Strategy for Malaria Control and Pre-Elimination 2011-2015). Support to the National Centre of Malariology, Parasitology, and Entomology (CMPE) is considered paramount importance. The Institut Pasteur du Laos, a Lao national institute for research and training on infectious diseases, is performing technical assistance for detection of malaria parasites in *Anopheles* mosquitoes to CMPE by the financial support of WHO.

Although information on the detection of human malaria parasites among the natural vector species using ELISA methods was reported in Laos (*Vythilingam et al*, 2005), there was no indication of molecular PCR-based methods available in country. Moreover, few attempts have been made in the Asia Pacific for in situ detection of Plasmodium spp. in vector species using PCR (Hasan et al, 2009; Bass et al, 2008). Very recently, malarial infections have been resurgent in the Southern provinces Lao P.D.R., hence, malaria control remains a high priority of the Lao government and WHO. The development of our Real-time PCR system to detect the parasitism in the naturally caught vectors in Lao PDR will be a very strong tool for supporting the control strategies of the malarial infections.

Aim of this technical service

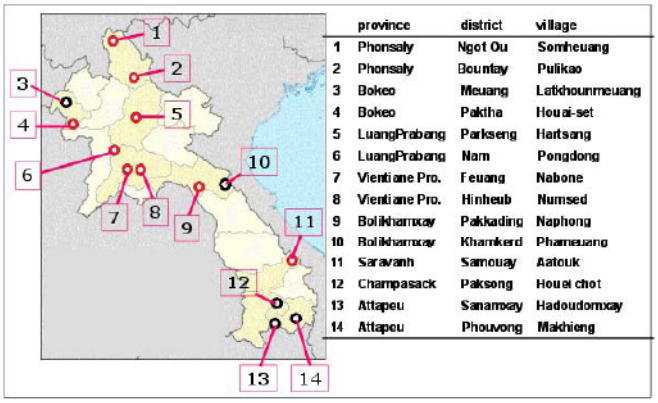
To confirm the distribution of *Plasmodium falciparum (Pf)* and *Plasmodium vivax (Pv)* in *Anopheline mosquitoes* in Lao PDR.

Materials & Methods

Mosquito collection

Between May to June 2012, mosquito collections were performed by CMPE. A total of 1515 *Anopheles* species were collected from 14 villages in 8 Provinces in Lao PDR (Figure 1). The mosquito collection time at the each site was at 18:00 to 24:00. All the specimens were morphologically identified on site and kept in liquid nitrogen during transfer to the institution. After that the specimens were all kept in -80°C prior to analysis.

Figure.1 The area map of mosquitoes collected



	province	district	village
1	Phonsaly	Ngot Ou	Somheuang
2	Phonsaly	Bountay	Pulikao
3	Bokeo	Meuang	Latkhounmeuang
4	Bokeo	Paktha	Houai-set
5	LuangPrabang	Parkseng	Hartsang
6	LuangPrabang	Nam	Pongdong
7	Vientiane Pro.	Feuang	Nabone
8	Vientiane Pro.	Hinheub	Numsed
9	Bolikhamxay	Pakkading	Naphong
10	Bolikhamxay	Khamkerd	Phameuang
11	Saravanh	Samouay	Aatouk
12	Champasack	Paksong	Houei chot
13	Attapeu	Sanamxay	Hadoudomxay
14	Attapeu	Phouvong	Makhieng

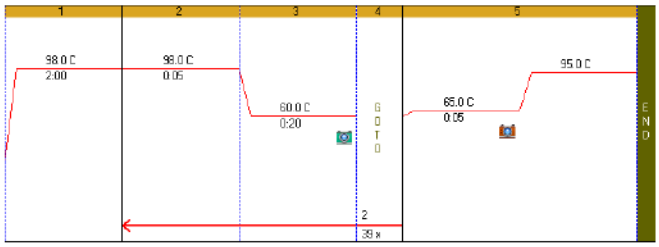
Extraction DNA from mosquitoes

Total genomic DNA was extracted from each mosquito using the commercial extraction kit (NucleoSpin Tissue, MACHEREY-NAGEL GmbH & Co. KG) according to the manufacture's instruction with minor modifications. Briefly, one mosquito was homogenized with 20ul of phosphate buffered saline (PBS) using a handy plastic pestle. After crushing the individual mosquito, the denaturing solution was added and incubated for 3 hour at 65°C. Every 30 minutes, sample was vigorously mixed by vortex. The sample solution was completely dissolved, the extraction was continued following the manufacture's instruction guide. Before screening assay, the extracted DNA was kept in 4°C for 1 or 2 days.

Real-time PCR assays for screening of *Plasmodium* species

For the screening assay, we targeted *Plasmodium* cytochrome b (cytb) gene. The primer set of screening are 5'-TGGAGTGGATGGTGTTTTAGA-3' as a forward primer, 5'-TTGCACCCCAATARCTCATTT-3' as a reverse primer designated by Dr. Didier MENARD at the Institut Pasteur du Cambodge. The Real-time PCR was performed with 10ul of Sso-Advanced SYBR supermix (Bio-Rad) along with 140nM of each primer in total volume 20ul. The screening template was two micro liters of genomic DNA extracted from mosquito, positive control and negative control. PCR was performed in a Mini-opticon (BioRad Laboratories, CA, USA) with initial denaturation at 95°C for 3min., followed by 40 cycles of denaturation at 95°C for 15 sec, annealing and extension at 60°C for 45 sec. Then continuously melting step was added for 65°C to 95°C, increment 0.5°C (Fig.2).

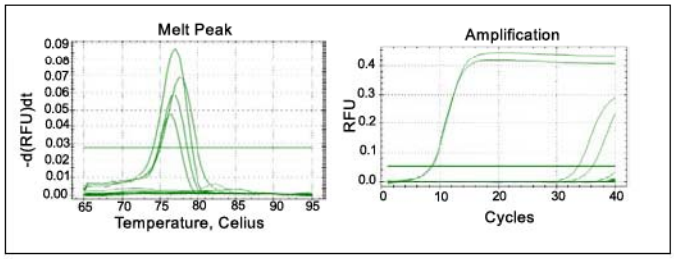
Figure.2 The cycling factors and temperature for screening real time PCR



Interpretation of results

The interpretation of the result depended on CFX manager version 3.1. In a valid run of screening via real-time PCR, a sample was considered suspected positive if a melting curve appeared at a melting peak temperature between 76.5°C to 78.5°C (Fig.3).

Figure 3. Melting peak and amplification profile for positive result



Identification of *Plasmodiumfalciparm (Pf)* and *Plasmodium vivax (Pv)*

Identification of the Pf and Pv species was done by nested PCR using specific primers. The screening PCR products of suspected positives were diluted 25, 50 and 100 times by distilled water. The nested PCR procedure was performed with Go-Taq (Promega, USA) and a DNA thermal cycler (iCycler, BioRad Laboratories, CA, USA). For Pf identification, thermal cycling with 35 cycles of 95°C 5 min for initial denaturation, then 95°C 30 sec, 65°C 30 sec and 72°C 45 sec. For Pv identification, the same protocol for Pf, but instead of annealing temperature 60°C. The PCR primers were

5'-AGATACATGCACGCAACAGG-3',
5'-TCATTTGACCCCATGGTAAGA-3', for Pf,
5'-TGCTACAGGTGCATCTTGTATTC-3',
5'-ATTTGTCCCCAAGGTAAAACG-3' for Pv.

The PCR products were separated on a 2% agarose gel electrophoresis, stained with Gel Red (Biotium, CA, USA) and bands were visualized by UV transillumination.

Results

The distribution of mosquitoes in area

Seven species of Anopheles were collected from 14 districts in 8 Provinces (Table 1); These species were *Anopheles.minimus* (An.min), *An.philippinensis (An.phi), An.maculatus* (An.mac), *An.jeyporiensis* (An jey), *An.dirus* (An.dir), *An.aconitus (An,aco), An.nivipes* (An.niv), and the numbers of collection were 602, 584, 162, 121, 12, 10 and 24, respectively. Notably, reduced numbers of Anopheles spp were collected in Champasak (33) and in Attapeu (21).

Table1. Sampling site and numbers of Anophelinae mosquito species

Province	District	Total	An. min	An. mac	An. phi	An. jey	An. dir	An. aco	An. niv
Phonsaly	Bountay	106	33	0	71	0	2	0	0
	Ngot Ou	103	52	0	51	0	0	0	0
Bokeo	Meuang	63	25	37	1	0	0	0	0
	Paktha	121	83	0	38	0	0	0	0
LuangPrabang	Nam	198	163	0	25	0	0	10	0
	Parksong	48	2	0	46	0	0	0	0
Vientiane Pro.	Hinheub	325	110	0	187	28	0	0	0
	Feuang	148	17	0	38	93	0	0	0
Bolikhamxay	Khamkerd	29	6	0	17	0	0	0	6
	Pakkading	158	44	0	95	0	1	0	18
Champasack	Paksong	33	1	25	7	0	0	0	0
Saravanh	Samouay	162	63	94	5	0	0	0	0
Attapeu	Phouvong	1	0	0	0	0	1	0	0
	Sanamxay	20	3	6	3	0	8	0	0
Total		1515	602	584	162	121	12	10	24

Evaluation of extraction methods from infected mosquitoes

Infected mosquitoes with Pf and Pv infections were kindly provided Dr. Ratawan Ubalee (AFRIMS Thailand) Four mosquitoes were extracted DNA by our method and confirmed to detect Plasmodium species using real-time screening PCR. All DNA from infected mosquitoes gave a positive amplification profile, and these melting temperatures were 77.0°C to 78.0°C as the same temperature as the positive control.

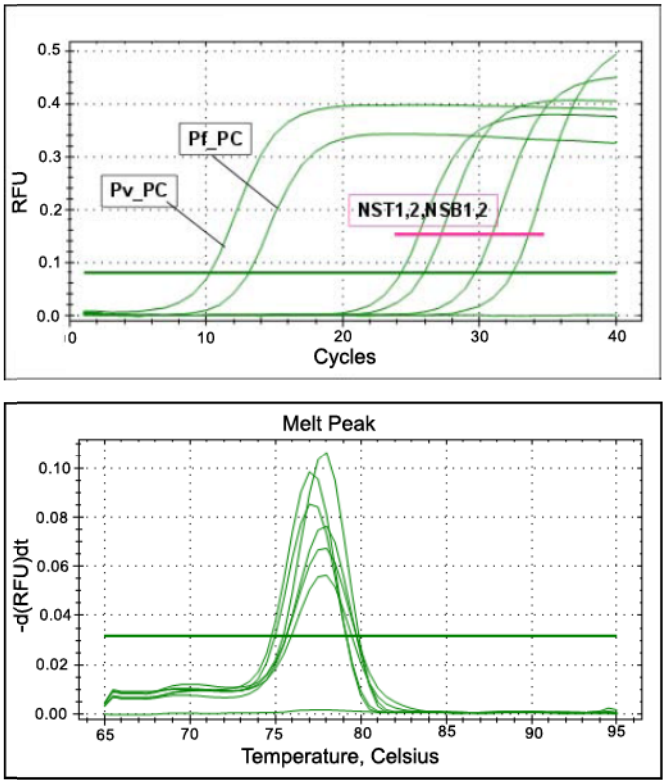


Figure.4 Real-time PCR profile for infected mosquitoes
NST1, 2, NSB1, 2 were mosquitoes DNA

The result of screening real-time PCR for Plasmodium species in mosquitoes

The real time PCR performed with specific primers for cytochrome b (cytb) gene in both Pf and Pv. Table 2 demonstrated that there are 38 positives out of 1515 mosquitoes (2.5%). The positive rates of the confirmed species of *An. minimus*, *An. maculatus*, *An. philippinensis*, *An. jeyporiensis* were 4.3%, 1.4%, 1.9%, 7.5%, respectively. In Aatouk village of Saravane Province, malaria positive rate among *An. minimus* and *An. philippinensis* were extremely high up to 28.6% (18/63), and 42.7% (3/7). Such high positive rates pushed us to repeat the experiments.

However, the subsequent analyses gave the same results demonstrating exceptionally high positive rates. Furthermore, very high prevalence of malaria in the village also supports this result. No *Plasmodium* species were detected from the other species. The malaria incidence rates in humans in the area is available (Jorgensen et al., 2010).

Table2. The distribution of *Plasmodium* positive in districts in Lao PDR

Province	District	all mosquitoes			Anopheles spp.						
		sum	+	%	An.min	An.mac	An.phi	An.jey	An.dir	An.aco	An.niv
Phonsaly	Bountay	106	3	2.83	1	2	0	0	0	0	0
	Ngot Ou	103	2	1.94	1	1	0	0	0	0	0
Bokeo	Meuang	63	0	0.00	0	0	0	0	0	0	0
	Paktha	121	3	2.48	1	2	0	0	0	0	0
LuangPrabang	Narn	198	2	1.01	2	0	0	0	0	0	0
	Parkseng	48	1	2.08	0	1	0	0	0	0	0
Vientiane Pro.	Hinheub	325	2	0.62	2	0	0	0	0	0	0
	Feuang	148	2	1.35	0	1	0	1	0	0	0
Bolikhamxay	Khamkerd	29	0	0.00	0	0	0	0	0	0	0
	Pakkading	158	2	1.27	1	1	0	0	0	0	0
Champasack	Paksong	33	0	0.00	0	0	0	0	0	0	0
Saravanh	Samouay	162	21	12.96	18	0	3	0	0	0	0
Attapeu	Phouvong	1	0	0.00	0	0	0	0	0	0	0
	Sanamxay	20	0	0.00	0	0	0	0	0	0	0
Total		1515	38	2.51	26	8	3	1	0	0	0

Identification of positive *Plasmodium parasites.*

For identification of Pv and Pf, we performed nested PCR using original primers in cytb of *Plasmodium* species. All PCR products analyzed sequencing and confirmed all species.

Figure 4. Identification of Pv and Pf by nested PCR.

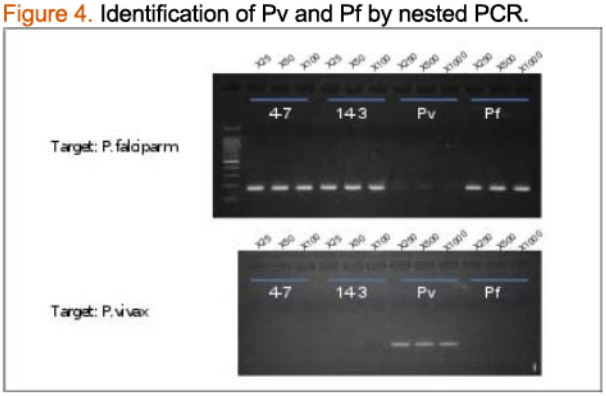


Table 3. showed the results of positive rates by each protozoan species in mosquitoes collected in country. Amongst the 38 mosquitoes, our analysis confirmed 34 cases of *P. falciparum* and 5 cases of *P. vivax independently*. Only one mixed positive case was confirmed in the results. The distribution of *P. falciparum-positive* mosquitoes was present in all provinces except Bolikhamxay, Dhampasack and Attapeu. The result of the last two provinces might come from the lack of significant the sample numbers. On the other hand, P. vivax positive mosquitoes were distributed in Phonsaly, Luang Prabang and Bolikhamxay Provinces.

Province	District	mosquitos			Pf	Pv
		numbers	+	%	positive	positive
Phonsaly	Bountay	106	3	2.83	2	1
	Ngot Ou	103	2	1.94	1	1
Bokeo	Meuang	63	0	0.00	0	0
	Paktha	121	3	2.48	3	0
LuangPrabang	Nam	198	2	1.01	*2	*1
	Parkseng	48	1	2.08	1	0
Vientiane Province	Hinheub	325	2	0.62	2	0
	Feuang	148	2	1.35	2	0
Bolikhamxay	Khamkerd	29	0	0.00	0	0
	Pakkading	158	2	1.27	0	2
Champasack	Paksong	33	0	0.00	0	0
Saravanh	Samouay	162	21	12.96	21	0
Attapeu	Phouvong	1	0	0.00	0	0
	Sanamxay	20	0	0.00	0	0
Total		1515	38	2.51	34	5

+: Number of positive; Pf: *Plasmodium falciparum*; Pv: *Prasmodium vivax*
* One mosquito had both species for Pf and Pv

Conclusion

A reliable PCR system, which detects *Plasmodium* species in anopheline mosquitoes was established in this study. Notably, Plasmodia were detected from 38 mosquitoes out of 1515 anophelines from the field collection samples by pattern recognition on the melting curve of the Real-time PCR system. DNA sequencing of the products of nested PCR system confirmed reliability of the methods. Amongst the mosquitoes, *A. minimus* was the most prevalent vector and nearly 70 % was confirmed at their positive specimens in Saravane Province. Plasmodium vivax positive mosquitoes were found in 3 provinces of Phonsaly, Luang Prabang, and Bolikhamxay in the study (Figure 6).

Conclusion

Modifying the rPCR system, simpler and more effective detection method on both malaria parasite and a vector species should be further develop, such as using the barcode biology system.

To grasp the present malaria vector situation more clearly, collections of the mosquitoes should be conduct in the remaining 9 Provinces with more intense collections in Saravane, Champasak and Attapeu Provinces.

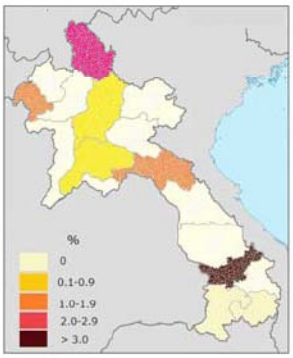
Importantly, a matched epidemiological analysis on human malaria prevalence and the composition of the Anopheles spp involved in transmission should be conducted in Saravane Province.

The mixed infection rate should be re-confirmed by the sampled species of No. 2.

Special efforts should be made for the collection of *An. dirus* because captures were very low. This species is known to be furtive and more intense collection efforts should be made in the Southern provinces experiencing a resurgence of malaria.

Possible nematoda (*W. bancrofti* filarial worms) and/or specific bacterial infections could also be examined in the samples.

Figure 6. The distribution of malaria positive rate among *Anophelinae* mosquitoes collected from eight provinces in LAO P.D.R



Mean *Plasmodium* positive rate: 2.5% (38/1515)

An. minimus	26/602
An. maculatus	8/584
An. philippinensis	3/162
An. jeyporiensis	1/121
An. dirus	0/12
An. aconitus	0/10
An. nivipes	0/24

Acknowledgement / Funding

We wish to thank Dr Didier MENARD, Molecular Epidemiology Unit, Institut Pasteur du Cambodge, who advised us on the screening real-time PCR method. Dr. Ratawan Ubalee, Armed Forces Research Institute of Medical Sciences (AFRIMS) Bangkok, kindly gave us the infected Anopheles mosquitoes. We thank Dr. Shigeyuki Kano and Dr. Moritoshi Iwagami for supporting this study and supplying the Plasmodium genomic DNA. We thank the staff of CMPE for collecting and morphological identification of mosquitoes in the field. This work was supported by the contract of WHO (reference 2012/266650-0.Reg File: Lao-12-MVP-003589) and in part by a Grant-in-Aid for Scientific Research (22256003) from the Ministry of Education, Culture, Sport, Science and Technology of Japan.

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Investigation on Schistosoma Mekongi in northern Laos

Coordinator:
Dr. Phonepadith KHATTIGNAVONG
Members of staff:
Dr. Lavy LORPHACHAN
Pheovaly SOUNDALA


Commemorative


Snail survey


Blood and stool collection

Schistosomiasis is a widely distributed intravascular trematode infection affecting >200 million people worldwide and is the second most important parasitic disease after malaria. This neglected disease, discovered in Laos in 1957, is prevalent in villages on the 4000 islands on the Mekong River in and around Khong Island, Champassak Province, the southern-most province of Lao PDR bordering the Kingdom of Cambodia. Parasite infection rates of up to 39% (184 egg positives / 473 villagers) were found in selected villages in this region (K. Boualy, 2007 CMPE Annual Report). People become infected when larval forms of the parasite (Cercariae) – released by freshwater snails (*Neotricula aperta*) – penetrate their skin during contact with infested water. Until recently, *Schistosoma mekongi* and its freshwater snail host, *N. aperta* were thought to be restricted to the Southern-most portions of the Mekong in Lao PDR. However, a recent snail survey in Khammoune province (400 km North of Khong Island) has shown *N. aperta* to inhabit certain rivers tributary to the Mekong. However, no *S. mekongi*-infected snails could be found during 2 years of survey. On the contrary, when subjected to *S. mekongi* in the laboratory, the Khammoune province-collected *N. aperta*, were shown to be susceptible to *S. mekongi* infections (Pusadee & Chanitma, 2012 (Malacological Investigations of Nam Theun 2 Hydroelectric Project in Khammouane Provience Phase II, Mahidol University). More alarmingly, Leshem et al., (*Emerging Infectious Disease* 2009 11, 1823) reported that twelve Israeli travelers acquired schistosomiasis in Laos 2002-2008 and 7 of them had acute schistosomiasis. These 7 patients were thought to be exposed to *S. mekongi* in the endemic areas of Southern Laos, whereas 4 patients were thought to be infected in the Vang Vieng, Northern Laos, which was considered hitherto devoid of S. mekongi infestation. Vong Vieng, nested on the Nam Song River, is one of the most popular tourist areas in Lao PDR for "water tubing" and other water sports attracting one hundred thousand tourists per year most of whom are backpackers < 30 years old.

Close scrutiny of the Leschem et al., 2009 study results seriously calls into question whether these four Israeli tourists were indeed infected by S. mekongi and whether Vong Vieng can be considered as a focus for S. mekongi infection and resultant schistosomiasis. In fact, Leschem et al. based their premise of "infectivity" solely on clinical symptoms and serological diagnostics (ELISA and immunoblots). The authors were unable to detect Schistosoma ova in the stool samples from any of the four patients thought to be infected in Vong Vieng. Furthermore, the authors themselves admit their premise that the four voyagers contracted schistosomisasis in Vong Vieng has several limitations: no ova evidence and possible cross-reactivity from other non-human Schistosoma spp.

Aim of the Project

To revisit Vong Vieng area and to investigate and determine if the local population harbors *Schistosoma mekongi* infections and if so what degree. To investigate and determine whether *Neotricula aperta* the obligate snail intermediate host of *S. mekongi* is present in the Nam Song River and its tributaries.

Results

Two snail survey comprised of 200 collection spots covering 17 locations along Nam Song River and its watershed. A total of 1155 snails (10 different genera) were collected and identified. No S. *mekongi* vector snails, *Neotricula aperta* were found in our collections. Of the snails collect cercarial shedding yielded three species of cercaria unrelated to mammalian schistosomes. In addation a total of 946 stool samples were collected from villagers (6-90 years-old with a mean of 27 years) from 36 villages in and around the Nam Song river watershed area. No *S. mekongi* eggs we found in the samples. However, 39% of the sampled population was to be positive for food-borne trematode *Opistorchis viverrini*, followed by *Strongyloides stercoralis* (17%), *Trichuris trichiura* (8%) and *Ascaris lumbricoides* (5%). Finally PCR on 100 random blood samples collected from villagers revealed no trace of *S. mekongi* DNA. This study demonstrates that there is no active transmission of *Schistosoma mekongi* in the Vang Vieng area.

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2 QUESTIONS ASKED TO THE C.F.O. OF IPL, **Antoine des GRAVIERS**

HOW DO YOU BREAK-EVEN FINANCIALLY AND HOW WILL YOU MAINTAIN THIS FINANCIAL EQUILIBRIUM?

"This is definitely a pertinent question!

Doing good science with publications, recruiting the best people and keeping the team together, investing in up to date technology and maintaining buildings and equipment, all of these tasks are the daily reality of Directors and Financial Directors in the Institut Pasteur International Network (IPIN).

To give you an global idea, the yearly budget of IPL is approximatively 2 millions USD among which around 40% of it is directly paid for by our partners of joint labs (salary of expatriate heads of labs mainly).

Trying to balance the finances of a new born institution, which doesn't generate funds nor receive recurrent subventions in a low income country is a brutally difficult task. It is nevertheless mandatory to make the Institut Pasteur du Laos sustainable.

With the Director we have been raising funds from the inception of the Institut Pasteur du Laos trying to be creative and using all the possible avenues. Some of the resources are quite classic like grants from institutional donors (Agence Française pour le Développement, France Expertise International, USA, ….) some other ideas are more original : yearly subvention from the institutions supporting each of the joint labs and covering a part of the general functioning costs (CRP Santé of Luxembourg, NCGM of Japan, Institut Pasteur Paris,…), some other ideas are being explored (establishment of a "fiduciary fund" from funds generated from philanthropy, or through the creation of for profit company,…)

My feelings are mixed, on the one hand of permanent enthusiasm and excitement to find solutions for funding of this institute and to see it growing and already generating concrete results, and on the other hand I realize the responsibility to leave a sustainable and healthy financial situation for the future of IPL."

HOW IS STRUCTURED THE ADM AND FIN DEPARTMENT AND HOW DO YOU SEE THE HR OF IPL ?

"Like in any other company Human Resources are a key factor.

The Financial and Administrative Department of IPL is structure in 4 entities (Accounting, Procurement/Logistic, Maintenance/ IT/Security, and Relations with Government) which encompass 20 people. All permanent staff are lao citizens.

The main objective of the administrative and support staff is to provide the best support possible to the scientific staff. It requires first to recruit the best people possible, then to train them, and finally to make them having the feeling to participate to a fantastic experience for the benefit of the health of their lao compatriots by actively supporting and backing up the scientists of the institute.

The administrative and support staff share the same values as the scientific staff in that their work is also contributing to the quality of our institute and in the long run to improve the health status of the Lao people.

We also try to provide to our administrative staff as much training as possible. For example, since 2012 we provided English lessons on site 4 times a week for all the staff. We financed a Master of Technology for our IT manager. Training on large equipment maintenance such as the HVAC systems also took place this year. Finally we provide first-aid training for our scientific staff and drivers.

My aim is that the administrative and support staff attain a high degree of autonomy!

They visited Institut Pasteur du Laos !

Mr. Francois HOLLANDE
President of the French Republic
05-NOV-2012

Mr. Francois Hollande and Dr. Paul Brey

Nguyen Thi Kim THIEN
Minister of Health of Vietnam
13-MAR-2013

In the center from left to right: Dr Paul Brey, Dr Kim Thien, Dr Ponmek Dalaloy, Pr. Bounkong Sihavong, Vice-Minister, MOH

Colonel Bountheum BANDAVONG
Director of Health Department of Lao People's Revolutionary Army
22-JAN-2013

From left to right: Dr Paul Brey, Col. Bountheum Bandavong

Mrs Pany YATHORTOU
President of the National Assembly of Lao PDR
03-APR-2013

In the center from left to right: Mrs. Pany Yathortou,Pr. Somock Kingsada, Vice-Minister, MOH, Dr. Malayvanh Lao

Mr. Jean-Claude JUNCKER
Luxembourg Prime Minister
04-NOV-2012

From left to right: Dr. Ponmek Dalaloy, Mr. Jean-Claude Juncker,Prof. Claude Muller, Mr. Peter Heiman

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