QA Discussion

The following highlights the questions and responses made by symposium participants at the end of all the sessions. Drs. Ron P. Marchand and Shusuke Nakazawa led the discussion encouraging participants to share thoughts and ideas on where to take the current research in Khanh Phu, while resolving the issues of species identification in the area. Individual names of those who commented are not included and where applicable, information is added to provide context in this summary.

The Khanh Phu Malaria Center faces the challenge of unambiguously identifying all the malaria species maintained in the area, especially in the advent of reports of past markers used for identifying *P. vivax* to also cross-react with many monkey malarias. The Khanh Phu Malaria Center would like to resolve the species identification issues by promptly analyzing samples obtained onsite using reliable molecular biology techniques.

The floor opened to discuss the immediate diagnostic capabilities of patients in the field using the Loop-mediated Isothermal Amplification (LAMP) method. As portable polymerase chain reaction (PCR) machines have been designed by the Centers for Disease Control and Prevention (CDC) in the past; could LAMP be a more suitable technique for field applications?

First comment:
LAMP is very simple and quite appropriate for field use. The procedure is very simple, so minimal training is required in the field. There are freeze dry kits developed for LAMP. LAMP can accurately distinguish species; LAMP for genus identification can be followed by a species specific, second LAMP step.

Comments on LAMP use in the field:
Using LAMP now would be a mistake, especially at a time when there appears to be a struggle with application of current traditional

techniques, ie). PCR, real-time PCR, and sequencing in determining species. LAMP offers a cheap, readily trainable system, but a solid foundation on current molecular techniques must be established, otherwise everyone involved would be wasting a lot of time and resources with inconclusive data. First, identify the parasites using the best available techniques, then move onto developing simpler techniques involving LAMP use in the field.

Additional comments to field development:
Since a field detection system that can be applied in the field is completely lacking in Khanh Phu, LAMP may just be the appropriate technology to develop along with establishing molecular applications to producing consistent data. This comment was prefaced with the current state of rapid detection kits in the field, where problems of false positives and false negatives with the use of ICT (immunochromatographic test) rapid detection kits in the field have been further developed to improve its technology.

Field applications may be required, but bad solutions that are cheap are not the way to resolve the present problems in Khanh Phu. Suggestions for suitable collaborators; although Khanh Phu may be considered remote, it is only 20-30km away from the Pasteur Institute with a lot of experience in many areas of molecular biology.

What is clear at present is that the current 18s rRNA primer sets to *P. vivax* detection is not capable of distinguishing among several known monkey malaria species. Generating primer sets for monkey malaria which can differentiate itself from human malaria is an immediate interest.

In Vietnam, PCR remains as the final confirmatory test applied to species identifications.

In Kyoto, molecular biologist and primatologists, entomologists, discussed which methods to use in the field (Khanh Phu?) to detect the parasites. As there are really no standards for field activities, we

are at a point in establishing standards of procedure.

Among the challenges in the field where the number of parasites are very little and multiple malaria species exist between human and monkey, it is important to be able to distinguish the parasites that have existed in humans, not only for diagnostic purpose.

LAMP is useful, but false positive results must be managed. PCR techniques can be further improved, but developing a panel to screen for all possible malaria species is needed right now.

Comment on how to work in parallel with recent malaria findings in the area:

Identifying what other parasites may exist in the Khanh Phu area may be of great interest. This will not only complement the malaria research, but also help understand the ecology of all diseases present in Khanh Phu beyond malaria, as there has not been a report identifying the impacts of other factors involved in the zoonoses. A global approach to understand the impact of certain factors to the ecology of disease could provide a better picture of the whole system in Khanh Phu. Addressing such a comprehensive approach may offer a way to obtain further support to studies that may have a broader impact.

Annual deworming intervention in the area was brought up as a method to understanding the re-infection rates of certain disease. This might be a great opportunity to carry out a survey right before an intervention to gain insight to all the microorganisms present in the area.

Expensive studies on transmission between humans and pet monkeys have been appearing in the literature, but not from Vietnam. Is this a topic that can be explored?

Malaria remains a problem here in the forest and is still a topic to

attract funding. How important is this threat involving monkey malaria in Khanh Phu? Are there other areas to focus our attention? This might be an opportunity to reevaluate our current understanding regarding persistence of malaria, especially with respect to zoonotic forest malaria.

Sarawak data system described, outbreaks are monitored intensively with integration of GPS data.
Does Vietnam have a systematic surveillance data or patient record reporting system?

Field data in Vietnam may still require collection efforts to be done individually for scientific validity.

Description of data management at Khanh Hoa General Hospital:
At present, Khanh Hoa General Hospital has a record of all severe malaria cases. Patients that have been admitted are properly recorded. There is a designated infectious disease section in the hospital dedicated to collecting patient data.

Response on the possibility of establishing a recording system:
Speaking from sixteen years of experience living in Vietnam, the Japanese funding agencies will need to come through with the time commitment and necessary funds/investments to make systematic data collection a possibility. GPS mapping of patients and carrying out extensive survey is quite possible. The Vietnamese personnel are very much interested in engaging in long term surveillance measures.

Additional comments:
In Binh Phuoc, 22 diseases are being reported to the central government with detailed diagnostics information. In some provinces, viral infections detected in patients are GPS mapped and sequence data obtained to follow the molecular evolution over time and space on pathogens of interest.

Description of a small scale surveillance system set up in Lao, PDR:

It is difficult to provide a nationwide surveillance system at this time, but based on a small scale Lao experience that began 3-4 years ago, handheld devices were implemented using village health volunteers (VHV) to carry out a household survey, covering a population of approximately 7000. There is potential to collaborate on setting up a similar system on a small scale first, and later consider expanding.

Discussion moved onto the next topic of interest involving antibodies detection methods in urine. What can be done with detecting antibodies in humans, beyond epidemiological relevance?

Comments on current capabilities with urine analyses:
The discrimination of species specific malaria is very difficult. Urine analyses and antibody detections are ideal in mass surveys, mainly to screen for various pathogens of concern. A realistic application is in screening school children on their health status as a monitoring tool. Using antibody detections for species identification remains to be investigated.

Further comments:
Serology studies are important. However, to know the serological responses to pathogens within a person requires understanding a whole lot, including not only the understanding of the disease, but also the dynamics of IgG and IgM in the blood in each specific population over time, and then translating the immunologic responses to the IgG and IgM dynamics found in the urine. Specific serological responses between populations are quite different even among the Vietnamese populations; IgG responses among the people in Khanh Hoa will be completely different from the people living in Ho Chi Minh City. Many challenges exist in validating the antibody-related responses to disease of interest.

Based from studies detecting malaria DNA in the urine, capturing the short window in which molecules of great importance such as DNA can be found excreted in the urine might offer an explanation as to why we see clinical variations in *P. knowlesi* infections among those

reported in Malaysia and other parts of Asia.

How can DNA be detected in the urine?
During the course of an infection, an immunological response follows with apoptosis of cells resulting in shedding of proteins and DNA. Cell contents of apoptosis passes through the permeable kidneys with urine in patients under stress and fever.

Single and multiple species infections, why is this an unusual effect to further understand the ecology surrounding disease transmission?
When considering an environment where all of these elements are available, the issue of surrounding mixed or single infection is quite strange.

Response to the above question:
Co-infections are common. What is so peculiar is that falciparum infections cannot be detected with knowlesi. P.v. + P.f./ P.v. + P.k. are common co-infections, but P.f. + P.k. has not been found. Even in positive mosquito salivary glands; with one exception of P.f+P.v. sample, most were of mixed infections. Could falciparum and knowlesi parasites exhibit competition? Parasite interactions still need to be investigated. Also, what we see as vivax, may not be entirely vivax. Could there be a monkey malaria species that has evolved in a way we have not identified yet? This is still unknown and brings attention to the interesting incident with detecting vivax in monkeys. For future studies *P. knowlesi* mono-infections must be detected in humans, the co-infection with vivax muddles the confirmatory testing, as we know now that the *P. vivax* markers also cross reacts with many monkey malarias.

Looking at the malaria phylogeny, *P. vivax* is much closer to monkey malarias, than *P. falciparum*.

Closing statement provided by Dr. Ron P. Marchand.
As a small group working on addressing some big questions, we will now be working to answer the questions addressed within the limited

resources available. Regarding identifications of species, doubts remain, but concerns will be worked out for solutions.

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