Plasmodium knowlesi in Malaysia: Bringing the Fifth Human Malaria Parasite from the Field to the Lab

Winston Churchill Travel Fellowship Report, 2011

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Aims and Introduction

Research Background and Fellowship Aims

I am a post-doctoral research scientist working at the Medical Research Council’s National Institute for Medical Research (NIMR), at Mill Hill in London. The aim of my work in the lab is to study the biology of human Malaria parasites, in particular focusing on the blood stages of the disease and how these parasites invade human red blood cells. To do this we use a range of techniques to look at the function of individual parasite genes and proteins in the hope that this will improve our understanding of how the parasite “works”, as well as hopefully identifying new targets for either vaccines or antimalarial drugs. In the last few years I have started working on the human/simian malaria parasite *P. knowlesi*, one of five species known to cause malaria in humans. This parasite is interesting for many reasons, because many of its traits make it much easier to study in the lab than other malaria species and also because it has recently been discovered to be a significant cause of human malaria cases in South-East Asia. My work in the lab has focused on developing a system to culture the parasites and we have recently become the first lab to successfully maintain a lab adapted *P. knowlesi* strain in culture with human blood. The aim of my fellowship will be to visit Malaysia to learn more about this malaria parasite in the field and also to attempt to collect clinical samples to attempt to adapt further lines to allow them to be studied in the lab. I have worked in malaria research for 7 years but before my travel fellowship, I had never done field work, never been to Africa or Asia and indeed never been to any country which actually has malaria.

Malaria Background

Malaria remains one of the most important infectious diseases, with 3.2 billion living at risk of the disease and between 350 and 500 million clinical cases a year (WHO, 2008). Over 60% of these cases and 80% of the reported deaths occur in sub-Saharan Africa, with over 1 million reported deaths per year here alone, mostly in children under five years old (WHO, 2008). Although Africa is the focal point of malaria transmission, it is a global problem and reported in over 107 countries, in South America, Asia as well as Africa. In addition to the toll in human lives, malaria also carries a significant economic burden, with some studies suggesting countries worst affected have economic growth rates reduced by as much as 1.3% per person per year, due to the mortality and morbidity, as well as the disease discouraging foreign investment and internal economic networks (Gallup and Sachs, 2001). There is currently no vaccine available for malaria and whilst malaria can be treated with antimalarial drugs, parasites have developed resistance to many of these, meaning the need for a vaccine and new antimalarials is very great indeed. Understanding the biology of this parasite is important to help us identify new targets for both vaccines and drugs.

Malaria is a disease caused by single-celled eukaryotic parasites of the *Plasmodium* genus. Species of malaria parasite can affect most vertebrate groups including birds, lizards and mammals, but typically each species has a highly specific host range, infecting only a few, or even just one, vertebrate host species. Previously there were considered four species of malaria parasites that affected humans; *Plasmodium falciparum*, *P. vivax*, *P. malariae* and *P. ovale*, but recent work has led to the addition of a fifth malaria parasite, *P. knowlesi*, to this list.

People contract malaria when an infected mosquito injects the parasite into the new human host whilst taking a blood meal. Once inside, the parasite travels first to the liver, where it invades and then grows
within the liver cells. Here it replicates rapidly, filling up the liver cell with thousands of invasive parasite cells known as merozoites. These tiny parasite cells (which are about 1/1000 of a millimetre in size) then released into the blood stream, and quickly start to invade red blood cells to initiate the blood stage of the infection. Once inside the red blood cell the parasite steadily grows, feeding on the haemoglobin within the cell, and eventually forms 8-32 new merozoite cells, which burst out and reinvade new red blood cells. It is this part of the infection that causes all the symptoms of the disease and indeed is the first point at which a patient can be diagnosed – normally by smearing and staining blood from the patient and examining microscopically. Disease results from the rapid growth of the parasite and destruction of red blood cells, resulting in things like anemia and low blood sugar. The body’s own immune reaction to the parasite also results in some of the worst complications of malaria, including the characteristic malaria fever.

Mosquitoes “catch” malaria when they take up the sexual stages present in an infected person’s blood stream, when taking a blood meal. It is only the female mosquito that takes a blood meal (as it needs extra protein to make eggs) and the shorter lived male mosquito lives on nectar alone. Importantly the mosquito is not just acting like a syringe and needle to transmit malaria by contact alone, but the parasite instead must undergo a series of development stages within the mosquito. This includes the parasites version of sexual reproduction and the parasite bursting out of the gut and then travelling up to its salivary gland, ready to be injected into a new human host. The complexity of the interactions between the parasite and its insect host means that malaria parasites can also only infect certain species of mosquito, and that, fortunately, most mosquitoes cannot transmit malaria. Malaria parasites affecting mammals (including all human malaria parasites) can only be spread by certain mosquito species within the Anopheles genus. Even some of the mosquitoes that are physically capable of being infected by malaria parasites make poor vectors for the disease. For example, a human malaria parasite may develop very well in a particular species of mosquito, but if that species mosquito only really likes biting chickens, or if it lives in an environment where humans rarely go (like in the treetops of a forest) then it will not be a very good vector for human malaria! It is for reasons like these that mean that the geographic range and intensity of malaria infections in humans is almost entirely dependent on the presence of mosquito vectors. It also means that the most successful methods for controlling malaria have been those targeting the mosquito vector, including draining the water they breed in, using bed nets to stop them from biting and spraying homes and breeding sites with insecticide.

An Introduction to *P. knowlesi*: Importance in the Lab and in the Field

Until recently *P. knowlesi* was considered a simian parasite, however molecular techniques have shown that *P. knowlesi* infections were being misdiagnosed as due to the more benign *P. malariae*. In one study, reassessment of 960 cases in Malaysian Borneo identified *P. knowlesi* in nearly a third, and it was the only malaria parasite present in the four fatal cases (Cox-Singh et. *GiemsA stained pictures of parasites:* These photos show red blood cells filled with merozoite parasite stages. The left cell is infected with *P. falciparum*, and the right with *P. knowlesi*. The small merozoites are dark blue with a purple dot where the nucleus is (taken by me at NIMR).
This study and many others identify *P. knowlesi* as a significant cause of malaria in south east-Asia. Although a small number of patients have been assessed, these analyses put the mortality rate caused by *P. knowlesi* on a par with that of the most deadly *P. falciparum*. In addition, as *P. knowlesi* can also naturally infects macaques in the wild, meaning it is the only human malaria with a significant alternative host to humans (it is generally described as a “zoonosis”, meaning an animal disease that can affect humans). This means unlike for other species, if it is eradicated in humans, it can still re-emerge from infections passed from macaques.

*P. knowlesi* is also a classic model to study malaria, with much of the early work on the mechanism of red cell invasion being performed using this parasite. The tiny invasive stages of the parasite known as merozoites are twice the size of those of *P. falciparum*. This is critical as these are amongst the smallest animal cells in existence, at about 1micron in diameter (or 1/1000 of a millimetre) which means much of their internal structure is not even visible with powerful microscopes. This stage of the parasite also stayed alive for a much longer time than in other malaria parasites meaning it was possible to capture the process of red blood cell invasion using video microscopy for the first time (Dvorak *et al.* 1975). These observations form the foundation of our current knowledge about red cell invasion by malaria parasite, but as the parasite could previously only be grown in the lab by infecting macaques, its use has dramatically declined over the years. In the UK in particular, very few labs maintain monkey colonies for ethical reasons and due to very strict guidelines, meaning worldwide only a handful of labs still work on the biology of this parasite. More recently it was shown that it could be grown in culture media *in vitro* (in culture flasks rather than inside an organism) using rhesus red blood cells (Kocken *et al.* 2002). It is also known that these cultured *P. knowlesi* parasites are easier to genetically manipulate than other species of malaria (Kocken *et al.* 2002), which is one of the main tools used to identify new functions for parasite genes. Whilst this is an excellent model to study malaria and also clinically relevant in its own right, its study still relied on access to macaque red blood cells, limiting its use to very few labs.

Having established this culture system in our own lab, over the last two years I have been able to adapt *P. knowlesi* parasite to grow in human cells. This is a huge step forward as it means there is no longer a requirement of access to macaque blood to undertake research on this parasite. The new focus on *P. knowlesi* as an emerging threat (Cox-Singh *et al.* 2008) and the completion of the *P. knowlesi* genome project (Pain *et al.* 2008), means that scientific and public interest in *P. knowlesi* as both a model and a bona-fide human malaria parasite is coming to the fore.
Itinerary

I planned to work with a collaborator, Dr Noor Rain Abdullah at the Institute for Medical Research (IMR) in Kuala Lumpur who undertakes field work to study Plasmodium knowlesi in Malaysia. I was able to join here and other research teams undertaking both lab work and on malaria field work around Malaysia. I also aimed to collect and preserve parasite infected blood from a range of sources. If these can later be adapted to grow in culture in the lab, they could be studied using a host of tools including genetic manipulation, DNA sequencing and drug screening. Culture of *P. knowlesi* in the laboratory has not yet been achieved in Malaysian labs so I also aimed to help them setup this technique and share my knowledge of molecular methods to study the parasite.

**Arrive in Kuala Lumpur (KL), Malaysia - 14th May 2011**

Start work at the labs in the Institute for Medical Research (IMR), KL- 18th May

Visit to the University of Malaya, KL – 26th May

Present talk at the IMR - 27th May

Talk at the University of Malaya, KL - 30th May

Field work in Kota Marudu, Sabah Borneo - 1st June- 10th June

Lab work in IMR, KL- 13th May- 15th May

Field work with Entomology to Lipis, 15th June-17th June

Lab work in IMR, KL- 20th June- 29th June

Field work joining Dengue survey team – 21st June

End of Fellowship period 29th June

Return back to UK- 14th July 2011
The Institute for Medical Research, Kuala Lumpur

Introduction to the IMR

For most of the time during my fellowship I was based at the Institute for Medical Research (IMR) in Kuala Lumpur, where I was hosted primarily by Dr. Noor Rain Abdullah from the Bioassay Unit. The IMR was one of the first research institutes to be built in the tropics, and was built in what was then British Malaya, to address the local problem of diseases like malaria and cholera. Established in 1900, the institute retained strong links to Institutes in the UK, like the London School of Hygiene and Tropical medicine over its more than one hundred year history. Whilst many of the original buildings remain from this time it has since been expanded and modernised, but has retained its remit to investigate the most serious public health problems of the nation – still including malaria but also extended to more “modern” diseases such as cancer.

Whilst similarly named, and both primarily government funded, the type of research carried out in the NIMR in the UK and the IMR in Malaysia are actually in many ways very different fields of research. The NIMR has a very wide focus examining anything, an in most cases working on understanding basic biology and fundamental research into how diseases work at the molecular level. This includes work on important classic model organisms such as fruit-flies to look at things like development, mouse models to look at immunology, as well as highly technical disciplines such as biophysics and protein structure to determine the precise structure of individual proteins. Still a developing nation Malaysia has many very pressing public health issues, and so its remit strongly focuses on epidemiology (the study of how disease spreads through the population), clinical diagnostics and research which can very quickly be applied to provide new tools or government policy to help fight disease. As such, the IMR is renowned for its clinical and field research expertise, with almost all labs focussing having either strong links to field working sites around peninsular or Malaysian Borneo or nearby Hospitals. An important implication of this is that the skill sets and expertise of researchers at the IMR and myself are very different, but highly complementary. This meant that I was able to learn a huge amount about the situation in the field and more clinical considerations of malaria and in turn was able to provide help with many of the molecular techniques from basic biological research which more recently are being applied to diagnostics and epidemiological studies. This also meant that we were able to think of many experiments to carry out which would be impossible if undertaken at either institute alone.
Lab Work in the Bioassay Unit

Dr. Noor Rain is in the Bioassay unit, within the Herbal medicine research centre (hmrc) headed by Dr Zakiah Ismail, whose focus is in particular to trial the medicinal properties of natural products. Whilst a focus of the lab is malaria, they also screen and run clinical trials on natural products to treat certain cancers, filariasis (a mosquito borne disease caused by a round worm) and the symptoms of dengue (a mosquito borne virus). On my first day I was introduced to the team and also the director of the institute, Dr. Shahnaz Murad. I was also introduced to Dr Shamilah Hisam, head of the Parasitology at the IMR, with whom I was able to have many discussions regarding the current situation with *P. knowlesi* infections in Malaysia. Dr Shamilah’s lab is involved in the diagnosis of suspected *P. knowlesi* cases, using PCR to identify the species of parasite using its DNA. She also introduced me to John Story a semi-retired veteran of the WHO, who had many fascinating stories about progress in fight against malaria in south-east Asia over the last 40 years or so.

During my time here I was mostly working with Masters students Azrina, Mieza and Mizan who carried out most of the culture work in the laboratories. A new laboratory facility had recently been completed, and this boasted very impressive cell culture rooms, and cutting edge equipment comparing favourably with facilities in the UK. Although well equipped, consumable reagents such as ingredients for media, used in culturing, remained a problem as normally these took several months to arrive once ordered!

An initial aim of the fellowship was to attempt to establish the culture of my lab adapted *P. knowlesi* parasites which I had worked on in the UK, to allow Dr Noor Rain’s lab to undertake experiments such as drug testing, and to hopefully aid in the adaptation of new lines. However, despite sending the samples required for this more than a fortnight before I arrived, two separate shipments were delayed and mishandled at customs resulting. When the samples finally arrived it was clear that they had thawed at some point during transit and hence were no longer viable. Whilst this was an early unexpected set back, we were able to instead concentrate on attempting to culture adapt new lines from samples. We were sent many samples of *P. knowlesi* infected blood from various nearby
Culturing parasite in a Microbiological cabinet: The hood produces helps keep the culture free from bacteria and fungus by creating a steady flow of filtered air.

In addition to the lab work I was also asked to give a presentation of my work in the UK to the institute. I gave a forty minute talk covering many aspects of my work in the UK, highlighting the people attended and I received a lot of interesting questions.

Entomology

In addition to spending time with Bioassay and Parasitology, I was also able to spend some time with Entomology, including undertaking field work with some of the teams. During my PhD I worked on the mosquito stages of a rodent malaria parasite and to do this we had a large insectary with colonies of several different species of mosquito. The IMR boasts an extremely large and impressive insectary so I was very keen to look around. Azian introduced me to Dr. Khadri, from the department of Entomology who was able to give me a tour of the insectary facilities. As well as species of mosquitoes that transmit malaria (those of the Anopheles genus) they also had species that transmit viruses like dengue, and also a species called *Toxorhynchites splendens*. The latter is an interesting species I had heard about before but never seen. It is a relative giant, with a body about 1.5cm long, but fortunately it does not drink blood! The adults survive just on nectar but it’s very large larvae feed on other mosquito larvae. This means that they can be used as a method of biological control for other disease spreading mosquitoes.

hospitals, and were quickly able to identify reasons why they had not been able to grow these for any period of time in the past. Many of these samples were very high but had been improperly stored, normally just frozen or placed in the fridge for up to a week. Without the addition of special preservative solutions, parasites cannot survive freezing and will also quickly die at colder temperatures. Also, some samples were from patients that had already been treated with anti-malarials, meaning many were already dead or dying before being introduced to culture conditions. Having identified these problems we were able to make recommendations to be passed on to the clinicians in the hospitals collecting these samples, which should hopefully drastically improve the quality of the starting samples, and thus increasing the chances of adapting a new clinical line to culture. Adapting culture protocols that I had previously used we were able to keep parasites going for up to a week in culture. Although this too eventually died out, we were able to make frozen stocks during this time which could enable us to have further attempts with these same samples in the UK.

Toxorynchites splendens: The giant mosquito that fortunately does not bite!
Mosquito rearing was carried out much as I have seen before. Basically eggs are put in a bowl of water until they hatch to form larvae. The larvae are then fed till they develop into pupae, at which point they are transferred to a small bowl of water in a netted cage where they can emerge as adults. Getting the next round of eggs is often the tricky bit, as most mosquitoes require a bloodmeal before laying eggs (hence why only females require a bloodmeal). Some species are very difficult to breed in the insectary so the IMR uses a special artificial mating technique to keep the colonies growing, the first time I have heard of this being used. As well as vectors of disease they also work on many other things including forensic entomology, which uses the type of bugs present to work out time of death, and also pest control, with a room full of various species of cockroach.

As well as Dr Khadri I also met with Dr. Nasni, Dr Rohanu and in addition to our various scientific discussions, we also discussed some of the logistical arrangements and difference between science in the UK and Malaysia. This was actually very interesting as logistics and bureaucracy have a major impact on the effectiveness of scientific research in both countries. One very noticeable difference is that whilst the labs in Malaysia are well equipped due to the complexities of ordering and procurement reagents can take several months to arrive, whereas systems have developed in the UK to the extent that I can order reagents and often expect them to arrive the next day. Other differences were less favourable on the UK, with the short contracts and poor career structure for post-doctoral researchers quite surprising for them when compared to Malaysia, where a shortage of trained post-doctoral researchers sees them highly valued.

University of Malaya

I was also invited to be shown around the Parasitology department of the University of Malaya in Kuala Lumpur, Malaysia’s oldest University by Professor Fong Mun Yik. I had previously worked with Professor Fong when he undertook a sabbatical working in the same lab as me at the NIMR. This was a great opportunity to both catch up and meet his fellow researchers, many of whom were also very interested in malaria and in particular *P. knowlesi*. The research at the University of Malaya was more like the kind of research carried out at our institute. So whilst there was a greater focus on work involving field isolates many researchers were involved in work to look at the basic biology of malaria parasites at a molecular level (i.e. studying the roles of individual parasite proteins) using the same techniques we
employ at the NIMR in the UK. I spoke to Prof. Fong at great length regarding the situation with \textit{P. knowlesi}, and many of his ongoing projects relating to this. Besides identifying many areas where we could potentially collaborate in the future he also spoke of some very interesting aspects of the problem that I was unaware of. Professor Fong and his colleagues were very welcoming and kind and I was later invited back to give a half hour presentation of my work which was well received.

**Field Work in Sabah, Borneo**

**Introduction Sabah Field Work**

The first field work trip I was able to take was to join Dr Noor Rain and her team in their trip to Sabah, Borneo. The trip was arranged to survey the area around a place called Kota Marudu for cases of human malaria, and to then obtain samples of parasites from any infected patients. Some samples would then be analysed in a field lab to test for signs of drug resistance, some would be processed to undertake genome sequencing and others would be used to attempt to initiate parasite cultures, so they could be studied once we had returned to the IMR. The study was specifically setup to look at \textit{P. falciparum} and \textit{P. vivax} infections, but \textit{P. knowlesi} infections were also known to be common in this area, providing an opportunity to obtain fresh samples of the type I would need for growing the parasite in the lab. We flew from the airport in Kuala Lumpur to Kota Kinabalu. Most of the equipment was taken with us as luggage, but fortunately some of the larger pieces, such as the centrifuge and incubator, were shipped separately across from the previous field work site. At the airport we were met by public health officials that work in and around Kota Marudu and they drove us up to our main base of operations. Kota Marudu, itself is a small town, with just a couple of hotels, restaurants and shops. We stayed in a clean but basic hotel, in the small town area and were able to set up a field lab in a large clinic nearby. Here we were given the use of an air-conditioned meeting room to set up the basic equipment we needed to process and culture samples. This included a 37\degree C incubator, required to incubate parasite cultures at body temperature, a centrifuge to spin down cells for washing and removing patient serum (which can otherwise cause clotting of the blood) and also the many disposable pipettes, flasks and special growth medium required to culture parasites. The team was composed of Dr Noor Rain, myself and three research assistants from Dr Noor Rain’s lab; Umi, Prem and Azrina. We were additionally joined by local ministry of health workers who monitor and help the villages in malaria hotspots throughout the year. These teams new the area intimately so could provide invaluable local knowledge. Not only were they superb off-road drivers, but they also knew the people of the villages and their own native language (which wasn’t Malay). As they monitored and help malaria management in each of the villages all year round, they also could advise us as to the best places to go. Although the team varied a bit day to day, most of the time the team consisted of Moizin, Saleh, Noor Saleh and Rahim. J’nita, a local
nurse, was also assigned to help us and was in charge of the blood pricks for the RDT and blood smears, as well as phlebotomy.

Although malaria is fairly common in this area of Borneo, its transmission is highly localised and predominately occurs in the small villages, “Kampung” in Malay, situated in the jungle or jungle fringe. So in order to catch as many cases of malaria, we aimed to drive out to these villages and screen as many people as were willing for malaria. Any found to be positive for malaria, would then be asked to provide a small sample of blood for our experiments (normally less than 10ml) and also provided with prompt treatment, either on site or transported to hospital. Participation was voluntary and it was made clear that they could withdraw at any point.

Borneo is home to a wide variety of ethnic groups and in this area it is largely the Dusun, an indigenous race, who live in these villages. The main occupation is rubber tapping and small scale subsistence farming in forest clearings. Unlike the Malay population which predominantly live in the town and city, the Dusun are mostly non-muslim and are culturally very distinct. Perhaps the strongest evidence of this was that pigs and dogs were everywhere in almost every village. The housing was highly variable in the villages, with some large sturdy wooden houses, but many simple constructions made of bamboo. The latter often were open on at least one side, and so, in the absence of a bednet, offered no protection from the night-time biting malaria vectors. The government also build brick housing and toilets in many of the larger villages and some also had access to the grid (overhead power lines in some cases ran quite deep into the jungle), or instead relied on generators. The size of these villages ranged from a few houses with one or two families to several hundred people.
Reaching the villages

The villages we visited were very remote and to reach them we relied heavily on the experience and skilled driving of the local public health team. Accidents on these sections of road are common, and so we always went out in at least two separate 4x4 pickup trucks with tow cables, in case one gets stuck or flipped on the narrow jungle tracks (which had happened on the previous trip). In most cases the village could be reached by about an hour drive along tarmac roads followed by a further hour or so trip along narrow and hilly mud tracks.

Whilst skilled drivers could navigate these roads whilst dry, if the weather turns to rain, which occurred almost every day, the roads quickly became impassable. Rain was also invariably torrential when it came, so the mud roads could be turned to foot deep rivers in a matter of minutes. This occurred on out first outing to a village when it became so bad that both cars had to turn back after about 30 minutes of driving. Whilst this caused us an unfortunate delay of a day, this was an everyday problem for the public health teams that work here. The village that we were trying to get to did in fact have several people positive for malaria and so this extra day not only delayed their treatment but it also increased opportunities for further transmission of the disease. It was clear that the inability to get supplies and respond quickly to new outbreaks of diseases here was a major reason why outbreaks of treatable diseases like malaria and cholera are so common and serious.

Malaria Diagnosis

Patients were diagnosed using two methods. The first is using blood films, which is the standard method to identify malaria infections as well as which species of malaria parasite is responsible, and the second is to use rapid diagnostic tests (RDTs). RDTs are a relatively new development in the fight against malaria and indeed are a very significant one. Traditionally, to diagnose malaria and to identify the species you must take a blood smear from the patient, stain it and then look for parasites with a microscope. This takes a lot of time, equipment and a skilled microscopist. I have worked on malaria for a long time now and spent much of that time staring down a microscope, and although I could identify a malaria positive sample, I would be very hard pushed to pin down the species (although I am better at this now). If the smear is taken in the hospital then this can easily be done in an hour, however if from the field, taking the smear back to the lab for staining and microscopy can often mean it is not until the next day that the patient can be brought in for treatment. Rapid diagnostic tests work using antibodies to the specific malaria parasites and function much like a home pregnancy test. You add blood, a couple of drops of buffer and depending on which bands appear you can identify whether there are malaria parasites there and also which species. The result from this can
Rapid Diagnostic tests: The RDT works like a pregnancy test if bands come up at either the Pan, Pv or Pf position the patient has malaria. The first on left is positive for P. vivax, the second, third, fifth and sixth from left are positive for P. falciparum.

be seen in less than 20 minutes and it takes no special expertise to carry it out or interpret. P. knowlesi has presented a unique problem in diagnosis as the parasites seen on blood smears look almost exactly like P. malariae, a benign human malaria species, leading it to invariably being misdiagnosed. Interestingly, the RDT, whilst not solving this problem, added further information to these cases. Whilst P. knowlesi looks like P. malariae, at a genetic level it is actually very similar to P. vivax (yet another human malaria parasite) and the antibodies used in the RDT which react to P. vivax also react to P. knowlesi! I had read a paper about this before leaving and was able to use this information to help identify cases of P. knowlesi. If diagnosed by microscopy as P. malariae and then by RDT as P. vivax, it was most probably P. knowlesi! To confirm P. knowlesi cases a DNA test is required (known as a nested PCR) so all cases of P. malariae are now routinely sent to diagnostic labs in the cities for final confirmation.

Screening for Malaria in the Kampung

On weekdays the aim was to arrive late afternoon so they could finish work first, and for larger villages word was sent out before we arrived to allow the villagers time to gather. The procedure depended on the size of the village but generally the screening was carried out on a few tables in an open but undercover room (it rained in almost every village we visited). There were 3 main stations for the patients. The first was for interviews and consent. Azrina, Umi and Dr Noor Rain were in charge of this. This simply entailed asking a few questions and explaining what we planned to do as well as taking details like name and age. As in addition to screening for malaria, samples would be taken for research the whole project had to first undergo strict ethical guidelines. If at any point they were unhappy they could decide not to take part (this included children who were scared or upset). After this they were
Screening patients for malaria: In these pictures I am helping J’nitoto screen patients for malaria in the villages (Kampung). Even in the smaller villages we quickly drew a large crowd!

taken a number and waited for J’nitota to do a finger prick (using a special disposable device) and then I took blood for RDT which Prem then setup, and Norsalleh made blood smears. Finally Rahim and Salleh announced the results of the tests so people could leave when the results were clear. The villagers were invariably very happy to take part and understood the importance of screening for malaria. For many I was also quite an oddity as very few westerners would have been to any of these villages, so for many I was the first white person they had ever seen. Besides my complexion and blond hair I was also very tall compared to the villagers, so I was constantly being watched by children and adults alike. Although this made me quite self-conscious, the inquisitive stares were also friendly and I was thanked by the few that spoke English, for coming so far to help them. The number screened per day varied from about 30 up to 150 in one of the larger villages, which in the extreme heat and humidity of the jungle was very hard work!

Taking blood for the RDT: Here is me taking blood for the RDT from a finger prick using a pipette. Note my very sweaty gloved hand!
Each day we also visited the hospital and with consent from the doctors and patients were able to take blood samples, from a number of patients who had come in with malaria over the previous day. The advantage of this is that all the malaria cases from the entire area, ended up coming to the same hospital and so we were able to see a far greater number of cases than we could possibly have found surveying each of the small villages. However, any patients admitted with malaria clearly must be treated quickly and so before we had been able to take blood samples the patients would have been treated with an antimalarial, in most cases using intra-venously administered artesunate. This meant that although most of the patients had relatively high numbers of parasites, which was good for drug assays and for attempting culturing, the antimalarial drugs had already started to kill the parasites, meaning those that remained were in poor condition and less likely to survive the switch to growth in culture flasks.

**Culturing and Work in the Field Lab**

Whilst I have been trained in sterile technique for culturing parasites and other cells, in a normal lab I would be doing this in special sterile hoods with a constant airflow designed to prevent bacteria and fungal spores from floating into the culture flasks, whilst they are opened to change media. The media used contains a mixture of nutrients like sugars and amino acids, along with human serum to provide everything the malaria parasites need to grow (in addition to uninfected human red blood cells to invade), however this is also the perfect environment for bacteria and fungi to grow. If a single bacterium or fungal spore gets into culture flasks like this, then they would very quickly outgrow the parasite and destroy the culture. In fact, despite my doubts, contaminations were not a problem, but there were many other difficulties of working in the field. The cryopreservative used to protect parasites during the freezing process actually freezes at temperature below zero, and so when we tried to use a domestic freezer to freeze the samples, we found they were not cold enough to freeze the samples. We were also latter unable to obtain dry ice to transport these samples frozen. In addition to freezing samples, infected red blood cells obtained from patients were used to test the effect of existing antimalarial drugs to look for drug resistance by culturing the parasites for short periods to determine whether the drugs kill the parasites at varying drug concentrations. Also attempts were made to adapt parasites to long term culture.
I was also able to help with the reading of blood smears to aid with the diagnosis of patients. To identify malaria parasites two types of smears are used, described as thick and thin. Thin provide the most detail and clearest morphology of parasites but the cells are less densely packed. Meanwhile parasites are less distinct on thick smears but allow you instead to look through hundreds of times more cells in the same time, making them far more sensitive, especially important in diagnosing people with relatively few parasites in their bloodstream. In the lab I routinely use thin smear, but although I have used thick smears, parasites are normally cultured at higher density and so these are rarely used by us. So for me just identifying parasites in these samples was challenging. Whilst I would not be confident enough to diagnose without a second opinion in the end I was much better able to identifying the species and scoring the infection.

**Summary of Data and Findings**

In total we were able to screen 359 people, including those from the hospital that had already been diagnosed with malaria. Of these people 22 people were found to be infected with malaria, 8 of which we detected in our village screenings, with 13 *P. falciparum*, 6 *P. vivax* and 3 *P. malariae*/*P. knowlesi* infections. We were able to get samples from most of these patients to carry out the experiments such as drug sensitivity, which were processed and completed at the IMR in the months following my departure. Although most cultured parasites did not survive for more than a day, a few lines grew for up to a week, which under such conditions was quite encouraging. The problems relating to freezing samples meant we were unable to return with frozen samples which may well have survived under laboratory conditions. It was also unexpectedly not possible to get hold of dry ice in Borneo, which would have be required to transport these samples whilst they were still frozen. A solution to this problem would be the purchase of a dry shipper, which we have used in our institute back in the UK. This is designed to carry frozen samples and can maintain a temperature of around -200°C for over a week. The advantage of this is that whilst it is “charged” using liquid nitrogen it does not contain any free liquid nitrogen and so can be transported on planes. We have already got plans to lend one of our own, and the IMR may indeed purchase one for this purpose.

We obtained samples of three patients with suspected infections of *P. knowlesi* (which were diagnosed as *P. malariae* by microscopy and *P. vivax* by RDT) and DNA samples were taken for each
of these to confirm the species of infection by PCR, upon return to the IMR. It was previously thought that *P. knowlesi* was only common infection in those who spent large amounts of time in the forest canopies, such as loggers who often slept in the forest and even in the tree tops. The patients suspected to have caught *P. knowlesi* in our case were the perfect example of how this restriction clearly does not apply - the patients were a 76 year old man, a 13 year old girl and a 9 year old boy. As none of these people are likely to be in the canopy, and are indeed would be in their villages or hoses at night, if the infections are caused by night-time biting mosquitoes infection is probably occurring within the village, and possibly within their homes.

On our last day we were joined by the Doctor in charge of public health in the area. He is based in Kudat, just to the north of Kota Marudu and he told us of that there was an extremely high rate of cases of *P. knowlesi* in this area. In 2009 there were two deaths from malaria in Kudat, and both of those were caused by *P. knowlesi*, with 125 cases occurring that year. He was very interested in my work in the UK and Dr. Noor Rain will hopefully be able to arrange future field work to look at this in more detail.

**Field work with the Entomology Team**

For my second field work trip I joined a team of 8 researchers from Entomology headed by Dr. Rohani. This was part of an on-going research project to study the malaria mosquito vectors present in a very remote area of peninsular Malaysia near a place called Lipis. This area was inhabited by an indigenous people known in Malay as the Orang Asli (aboriginals). This area is inhabited by the Senoi, who live a simple life primarily as hunter gatherers but also some small scale farming. Rather sadly they also now seem to rely quite heavily on rations provided by the government, perhaps as forests are encroached by plantations and many of the animals they hunt become endangered. As in Borneo, with isolation comes risk of malaria, and so transmission rates remain high in the Orang Asli villagers. Interestingly whilst other human malaria is prevalent, there is thought to be little or no transmission of *P. knowlesi*. One explanation proposed to me for this was that this is because there are few macaques in the area as they are hunted by the Senoi.
We travelled in two 4x4 vehicles from the IMR loaded up with all the supplies we would need for our 3-day trip. This included everything we could need for the trip like tents, camp beds, a generator, fuel and enough food for the whole group. We first travelled on the highways for about 3 hours north-east before turning off onto an inconspicuous side road. From here on in there was only dirt tracks, winding through thickening jungle, with the roads far worse than where we had visited in Sabah, Borneo. Many had deep ditches running through the middle caused by rain run-off and despite it remaining dry, it was a very bumpy ride. Fortunately the drivers were also very experienced and skilled and after about 3 hours of further driving we reached the Senoi village, called Bukit Long. Although the villagers live in traditional bamboo huts, the government had built several permanent buildings in each of the villages. These are generally shunned by the locals as they prefer houses that can easily be taken down and relocated (for example it is tradition to abandon a house if someone dies there). We were allowed to stay in one of the empty concrete buildings in Bukit Long by the chief. Although still basic it had a sink in the kitchen, two bedrooms and a drop toilet as well as a generator already hooked up. They had also bought a camping stove with them and an ice box for meat, so even here I enjoyed very good food!

The team carry out two main projects within this area. The first is known as the bare-leg assay (abbreviated to the B.L.A), where essentially you sit on a chair for several hours at night with all skin covered other than your legs. Then using a torch to regularly inspect your legs, you capture any mosquito attempting to bite in a small glass vial. This technique is demands your complete attention and was actually quite risky. Whilst I was taking antimalarials, many of the other team members undertake field work so regularly that they cannot constantly take them as prophylaxis. The technique although simple, is actually very powerful as it allows you to determine which species of mosquito bite humans at specific times and locations in the village, and back in the lab it can also be determined whether they are infected with malaria or other diseases. In the past this has enabled them to determine that the peak time for the main vectors for malaria to bite are between 10 and 11 at night. On the night we undertook this assay it had started to rain, and despite waiting for several hours I did not catch a single mosquito! Fortunately the other members of the team and some of the villagers were more successful.
The second technique was the river larval survey and proved the most adventurous of all my experiences in Malaysia (but also one of the most enjoyable). To breed, mosquitoes like still water, and so the pools of water that occur next to rivers provide ideal breeding sites. The teams project aims to survey these pools to identify which mosquito species breed in these areas at different points along the course of a river and at different times of the year. Myself and four other member of the team set off to the nearby river that they had focused on for their research. We took with us a radio each, a machete, a GPS tracker and ladle and pipette for collecting the mosquito larvae. All electrical equipment including my camera was wrapped in plastic bags and elastic bands, because it was presumed that we would get very wet. Starting near the top of the course we slowly worked our way down the river sampling the various pools of water to look for larvae. If found the larvae were collected in tubs and the genus identified and noted down along with the GPS location for the pool. Identifying the species of mosquito can only reliably carried out on adult mosquitoes so these would be taken back to the lab and allowed to develop to adults. The species could then be identified and if enough collected, used to establish mosquito colonies in the lab.

The river itself was surrounded by thick jungle and was in a steep valley, and so most of the time we walked in the rocky shallows along the shore and at times by walking straight down the middle. Although generally the water stayed below shoulder level, it was very fast flowing meaning when shallow, meaning keeping a footing was often difficult. Fortunately the shallow water meant primarily it was only my dignity at risk, for most of the time. We also had to climb down waterfalls, and over dams created by fallen trees and whilst it was hard work, Zamri, the experienced guide from the IMR led the way finding the safest route down the river. We followed the course of the river for about 4 kilometres taking nearly 4 hours in total. When we finally ascended the steep valley side, we realised that we had climbed up the wrong bank and could see the village again far on the other side of the valley!

Besides the research I also had a unique insight into the village life, although
language again became a huge barrier. I was even more of a novelty here than in Borneo and as always it was the children who were most obvious with their curiosity, gathering in the evening outside our building to watch us. Despite living an extremely isolated life, the influence of the outside was evident in many ways, in particular the rock band that started playing from one of the bamboo huts every night. Complete with electric guitars and drum kit they played many great versions of the songs I had been hearing on the radio, only stopping when the generators eventually spluttered and ran out of petrol for the night. I was also able to meet some of the teachers at the nearby school. This again was a permanent building built by the government- and actually represented the most expensive school in all Malaysia. The area was so remote that almost all the building materials had to be shipped in by helicopter! The Senoi people are protected by the government and as an outsider I was incredibly lucky to have permission to join the teams in the Orang Asli settlements. The field work was also arranged at short noticed and seemingly mostly for my benefit so I was very glad to be offered the chance to join them by Dr. Rohani.
Conclusions and Perspectives

The fellowship provided an unparalleled opportunity to learn about tropical medicine first hand, and in particular shed light on what seems to be a serious and newly emerging disease in humans. Thanks to the generosity of my hosts I was able to meet and work with some of the top researchers in the fields of tropical medicine and entomology and learnt a wealth of information highly relevant to my research on malaria. I was also able to join two different teams carrying out malaria field work both in the Peninsular and Malaysian Borneo. During this time I was able to collect a number of samples which could be used in numerous ways to help our research in the UK. Whilst I was unable to adapt a new parasite line to culture in the labs at the IMR, many of the samples collected will allow me to try many other conditions back in the UK. We currently have collaborations with specialised genome sequencing laboratories so we may also be able to use some of these samples to sequence the entire genome of a clinical isolate. This has not previously been done for any clinical sample of *P. knowlesi* and may reveal unique gene differences between clinical and laboratory reference parasite lines.

Whilst previous work had highlighted that *P. knowlesi* may become a serious threat in some areas of south-east Asia, the paucity of publications on this malaria compared to other species meant it was hard to get a clear picture of the actual situation. From everyone I spoke to working in public health in both Peninsular and Malaysian Borneo it is seen as an extremely important emerging threat. This is not only because Indeed upon my return a recent survey published on childhood malaria in Kudat in Sabah (the region just north of where I visited) showed that over half of hospital cases of malaria in children were caused by *P. knowlesi*, and this was an even greater proportion in adults (Barber *et al.*

*End of the day: Travelling back from field work in Borneo*
One explanation for *P. knowlesi* becoming dominant in these areas suggested in this study is the effectiveness of intervention against other human malaria parasites. It is thought that immunity to human malaria parasites such as *P. vivax* can also provide some protection against *P. knowlesi*. However as malaria control methods result in dropping cases of *P. falciparum* and *P. vivax*, this cross-species immunity would also decline, thus resulting in increased susceptibility to *P. knowlesi*. This may be further compounded by humans and macaques (which provide act as a reservoir host in this case) coming into closer contact as many areas of Malaysia are deforested. Malaysia is currently in the pre-elimination phase of its eradication program, with ambitious plans to eradicate malaria entirely within the next 10 years. The two greatest threats to this aim are the large number of imported cases brought in by immigrants less willing to seek treatment, for fear of deportation, and *P. knowlesi* where the disease would also have to be controlled in a large reservoir population of macaques. It was also mentioned to me that more serious steps could be considered, such as the culling, or relocation of macaque populations living near to humans in areas with high levels of *P. knowlesi*. It is only with greater understanding of how this parasite is transmitted, and in particular behaviour of its primary mosquito vector that such tactics can be avoided.

One of the most important aspects of the fellowship was that it enabled me to meet researchers in a wide variety of fields, including those specialising in diagnostics, epidemiology, and clinical aspects of malaria *P. knowlesi* as well as those studying their mosquito vector. Since my return I have been in contact with many of these already and we hope this should continue for many years to come.

Since arriving back in the UK I have been accepted to present my work on *P. knowlesi* at the Molecular Parasitology Conference in Woods Hole, in the USA. This was paid for by UK institute, and whilst primarily focused on my lab work in the UK, it also included a slide describing some of the work carried out during the fellowship. This talk was met with a great response and I was subsequently asked to submit an abstract to speak at a conference in Australia early 2012 by the organisers. I additionally won the speakers prize for the US conference, winning an invitation to speak at the American Society for Tropical Medicine and Hygiene (ASTMH) annual meeting in Philadelphia in December, with all expenses paid. We hope to publish some of our work on *P. knowlesi* in the next year but this will hopefully be the first of many on this subject, with collaborators in Malaysia also hopefully playing a part in this. Due to the recent success of my work in *P. knowlesi* it is likely, if all goes well, that this will become my niche within malaria research. This fellowship will be invaluable not only in the knowledge, contacts and research materials that it provided but also will be superb support for the next steps in my career which will be to look for further research funding in both grants and fellowships.

On a personal level I found that the trip expanded my horizons immensely, with the experience unlike anything I had ever done before. Despite being terrible at languages in school I found myself confidently walking into provincial cafes.
and ordering entirely in Malay often earning broad smiles or exclamations of “pandi” (clever!), or making small talk with taxi drivers in a half and half mix of Malay and English. Much of this confidence came directly as a result of how well I was looked after and guided by my hosts. During the first few weeks of my fellowship I was joined by Noor Azian Yusuf, who is currently studying for a PhD at the NIMR, but is on leave from her position at the IMR. Thanks to her and her family I was very quickly able to feel at home in a very different culture.

Another interesting observation I made in my travel in Malaysia was that almost every senior member of staff at the IMR had a one point studied in the UK, either as a Masters student or for a PhD. It was also clear that the quality of research and education in tropical medicine undertaken in the UK is held in great esteem even today. Many of the young researchers I met were very eager to here of what research is like in the UK, and hopefully several will apply for funding for PhD or further training in UK institutions as a result. Despite a shift away from investment in education over recent years it is clear that Britain can still lead in medical research, and this is something that Britain should be very proud to export across the world.

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Malaysia is culturally very different from the UK and my colleague Noor Azian Yusuf acted as an invaluable guide and “agent” during my time in Malaysia. Thanks to her help I was able to make the most of my trip, understanding customs and to small extent language, as well as identifying the best food to eat. I would also like to thank her family for inviting out to many different places including taking me to the Cameroon Highlands, and an amazing family feast in their home town, and for being so hospitable. I would also like to thank the MRC and my bosses at the NIMR, Mike Blackman and Tony Holder, for supporting my fellowship and allowing me to take time off from my normal lab work. Finally, thank you to the Winston Churchill Memorial Trust for this amazing opportunity and to all the staff there who helped ensure it ran so smoothly.
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