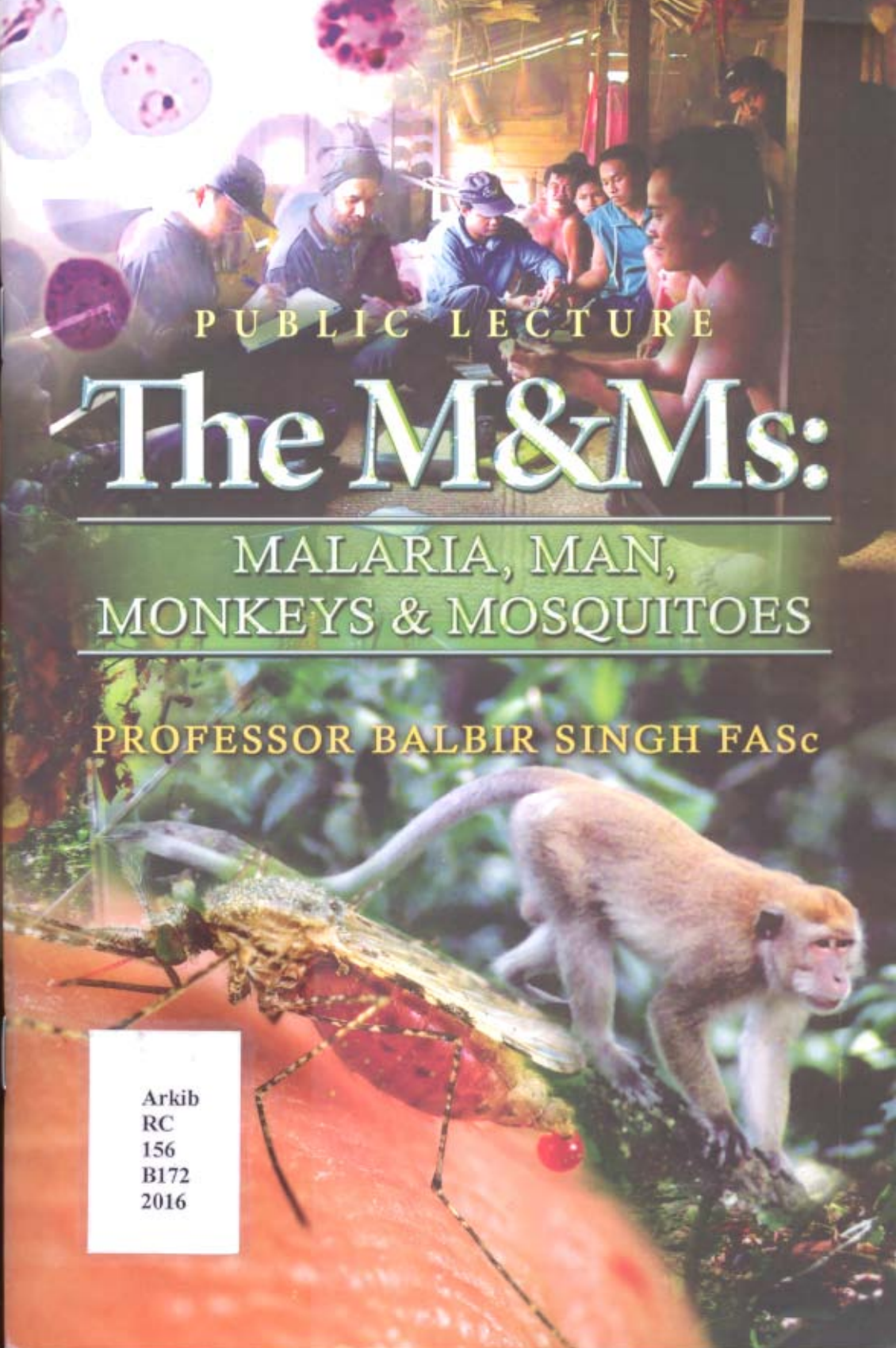

PUBLIC LECTURE

The M&Ms:

MALARIA, MAN, MONKEYS & MOSQUITOES

PROFESSOR BALBIR SINGH FASc

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MALARIA, MAN, MONKEYS & MOSQUITOES

a Public Lecture
by

Professor Dr Balbir Singh FASc
Malaria Research Centre, Universiti Malaysia Sarawak

Thursday, 18th August 2016

PUSTAKA Negeri Sarawak
Jalan Pustaka, Petra Jaya
93050 Kuching
Sarawak, Malaysia

FOREWORD

The Faculty of Medicine and Health Sciences, Universiti Malaysia Sarawak is committed to producing competent and compassionate graduates to meet the health care needs of the community through educational excellence and research of international standards. Apart from involvement in producing graduates through teaching, our lecturers need to do research to discover new knowledge, methods and treatment in the medical and health care fields, in the hope that these will benefit the community around us.

Universiti Malaysia Sarawak through the Public Lectures give the researchers the opportunity to share their new discoveries, methods or treatment with the public.

I would like to congratulate Professor Dr Balbir Singh for this Public Lecture entitled "The M&Ms: Malaria, Man, Monkeys & Mosquitoes". One main discovery that Professor Dr Balbir Singh and his team made was in the year 2004 where *Plasmodium knowlesi* was found to be a monkey malaria parasite that can cause malaria in humans. This discovery has contributed for a better understanding of malaria and subsequently to changes in the latest management of patients infected by these parasites.

Professor Dr. Ahmad Hata Rasit
Dean
Faculty of Medicine & Health Sciences
Universiti Malaysia Sarawak

Figure 1. The 'malaria detectives' at the Malaria Research Centre, UNIMAS in 2006. Sitting from left to right: Cyrus Daneshvar, Janet Cox-Singh and Balbir Singh. Standing from left to right: Paul Divis, Sunita Shamsul, Angela Siner, Lau Hui Chong, Sophia Lau, Siti Khatijah Zakaria, Roynston Julin and Lee Kim Sung.

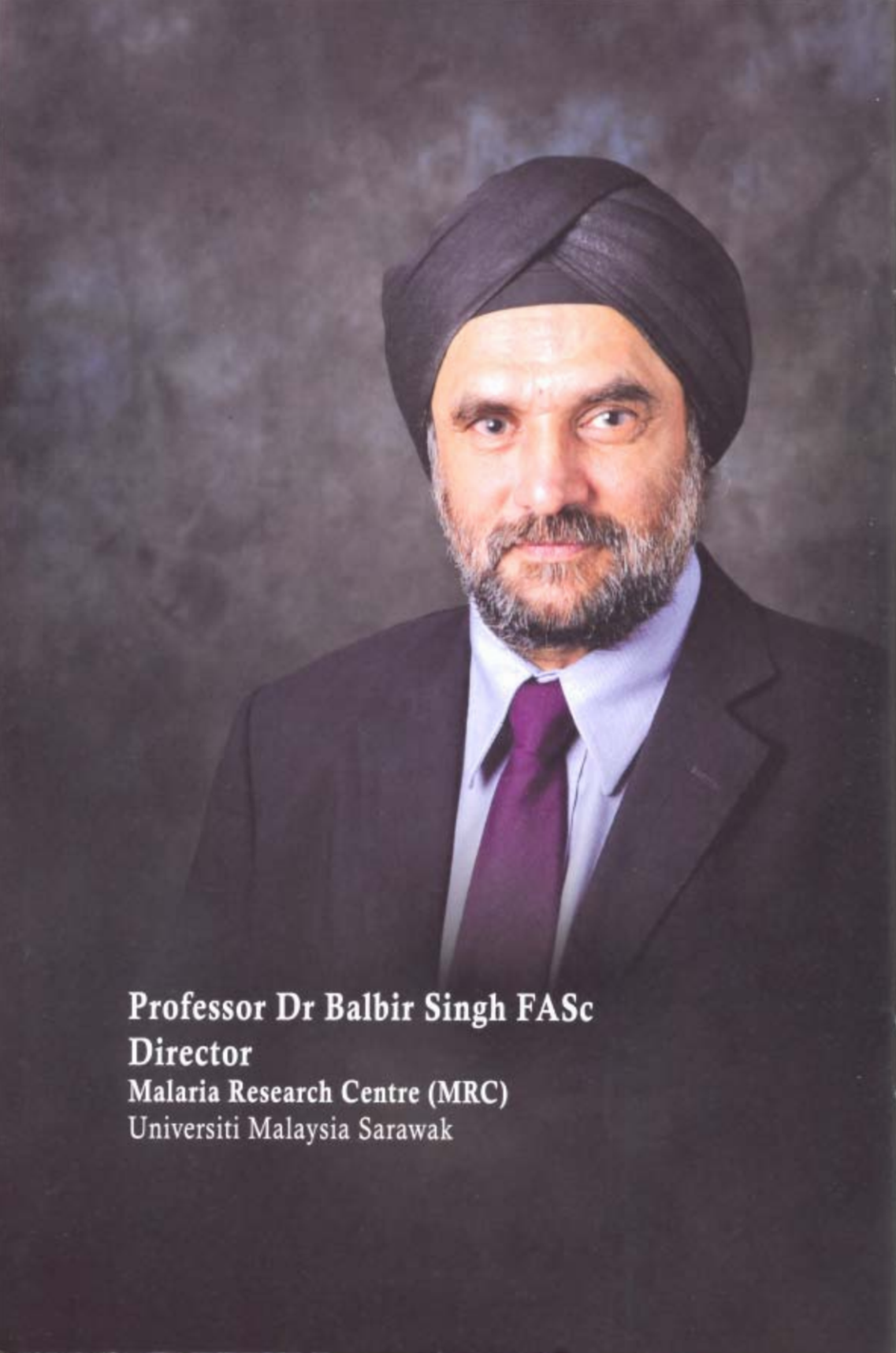
PREFACE

The work I undertook as a scientist since I began my postdoctoral research in 1984 primarily involved the M&Ms: Malaria, Man, Monkeys and Mosquitoes. I could add another 3 Ms, which are Malaysia and Molecular Methods, but the title of my talk is long enough. A scientist is an investigator, and as someone who has investigated malaria for over three decades, I consider myself as a malaria detective. Every successful detective has to have an efficient team. I could not have done my research without the assistance of Dr Janet Cox-Singh and our team of fellow scientists, research students and laboratory staff who spent many hours in the laboratory and in the field, and helped us answer the various research questions that were posed. I am very proud of the people that I have had the pleasure of working with from Universiti Malaysia Sarawak (UNIMAS), especially the original team of malaria detectives (Figure 1) that did the pioneering work on molecular and clinical aspects of knowlesi malaria, and I will forever be indebted to them.

I am also grateful to staff of the State Health Departments and Hospitals in Sarawak, Sabah and Pahang, and our collaborators from Malaysia and overseas, who assisted us in conducting our multi-disciplinary research. My team and I are thankful to the higher management and other staff of UNIMAS for their unstinting support which enabled us to conduct our research. Finally, I am indebted and grateful for the assistance of the malaria patients who willingly donated their blood as well as the inhabitants of the longhouses in Sarawak who co-operated fully and helped us in our field studies.

Acknowledgements

I have already thanked many people in the preface section for all their assistance. I would like to thank Dr Kevin Palmer, who first suggested that we study *P. malariae* in Sarawak; Dr Angela Siner, Dr Khatijah Yaman, Thamayanthi Nada Raja, Joshua Ang, Liew Sze Tze, Anna Hu, Khamisah Abdul Kadir, Dyg Shuaisah and Ismandy Kria for their tireless work at the MRC, and staff at UNIMAS for all the years of support and for the recognition of our work by establishing the Malaria Research Centre in 2006. I am also grateful for research grants from UNIMAS, from the Ministry of Science and Technology, the Ministry of Higher Education and from the Wellcome Trust, United Kingdom. Finally, I am thankful to the Sarawak Biodiversity Council and the Forestry Department for the permits which allowed us to undertake our research.



Professor Dr Balbir Singh FASc
Director
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ABOUT THE PRESENTER

Professor Dr Balbir Singh is the founding Director of the Malaria Research Centre (MRC), Universiti Malaysia Sarawak (UNIMAS). He spent 18 years in his hometown, Segamat, in Johore State, before spending a similar duration in England. He received his BSc and MSc degrees (1976-1980) from the University of Liverpool, where he subsequently pursued his PhD studies after being awarded a university scholarship. His research interest in malaria began when he joined the Liverpool School of Tropical Medicine (LSTM) in 1984 as a Postdoctoral Senior Research Assistant. Subsequently, he received a Beit Medical Fellowship in 1988 to work on molecular and biological aspects of malaria at the LSTM. He returned to Malaysia in 1992 to take up a lectureship in Medical Parasitology at Universiti Sains Malaysia and moved to Universiti Malaysia Sarawak in 1999.

Prof Balbir has been using molecular tools to study malaria for almost three decades. Research at the MRC, UNIMAS focuses on the epidemiology, pathogenesis, evolution and population genetics of malaria parasites. His research team made a landmark discovery in 2004 that *Plasmodium knowlesi*, a monkey malaria

parasite, was causing malaria in humans. They subsequently made several other key discoveries which have highlighted knowlesi malaria as a life-threatening zoonotic disease that is prevalent throughout Southeast Asia.

Prof Balbir has received grants totalling RM 6.85 million from local and international funding bodies such as the World Health Organisation (WHO) and the Wellcome Trust. He has published in leading international journals including *The Lancet* and *Clinical Microbiology Reviews* with 2,929 citations and has an *h*-index of 27. His team's work on knowlesi malaria has attracted considerable media attention and was featured in a documentary by ABC Australia and in a news report by Al Jazeera. Prof Balbir has been invited to speak at numerous conferences in Europe, USA, Canada and Australasia, to review research grant applications from the UK and Australia, and to review manuscripts for publication in various journals including *Lancet Infectious Diseases* and *Clinical Infectious Diseases.* In addition, he was an Advisor to WHO (2011), was a member of the editorial board of the *Malaria Journal* (2002-2009), and served as local secretary for Malaysia for the Royal Society of Tropical Medicine and Hygiene, UK (1994-2007). His contributions to Science in Malaysia were recognised by the Academy of Sciences Malaysia when they selected him as one of the Top Research Scientists Malaysia in 2012 and invited him to become a Fellow of the Academy of Sciences Malaysia in 2015.

THE M&Ms: MALARIA, MAN, MONKEYS & MOSQUITOES

INTRODUCTION

Malaria is caused by a parasite called *Plasmodium,* which is larger than a virus and a bacterium. There are over 150 different species of Plasmodium, and in general, they tend to be host-specific; the five malaria parasite species that are found in humans (*P. falciparum, P. vivax, P. ovale* and *P. malariae*) only infect humans and cannot be transmitted from humans to animals by mosquitoes. Malaria is transmitted by Anopheline mosquitoes and when an infected mosquito feeds for blood, it also injects malaria parasites with its saliva into the blood stream. These initially travel to the liver, where they develop and multiply inside a cell. An infected person shows no clinical signs and symptoms when the parasites are developing in the liver, and this period can last from one week to months and, at times, even years. The liver cell bursts and releases thousands of parasites which invade red blood cells. Within these cells they are protected from the person's immune response and they can develop and multiply.

For the human malarias, this multiplication cycle is dependent on the species of Plasmodium and lasts for 48 or 72 hours. In some patients, there is a fever peak every two or three days which corresponds to the multiplication cycle of the type of malaria parasite in the blood. As the parasite multiplies the number of malaria parasites increase in the blood and, if left untreated, can lead to severe manifestations and even death. As the signs and symptoms of malaria are quite non-specific, and someone with a viral or bacterial infection can have similar symptoms, a blood test is usually undertaken to confirm that the signs and symptoms are due to malaria parasites. This test involves obtaining a small drop of blood which is spread on a slide and examined under the microscope after it has been stained with a dye. Experienced laboratory technologists can identify the different species of malaria parasites based on a number of characteristics such as the presence of dots or stippling, and whether the infected red blood cells are enlarged. A more sensitive but expensive method of diagnosis involves molecular detection, where parasite DNA is detected, which can only be conducted in specialised laboratories such as the Malaria Research Centre at UNIMAS.

Investigation of Atypical Malaria in Kapit Division

For many years, the main cause of human malaria in Sarawak has been *P. vivax*, followed by *P. falciparum* and *P. malariae*. However, the scenario in Kapit Division was atypical compared with the data for Sarawak in that after *P. vivax* malaria, *P. malariae* cases were more common than *P. falciparum* malaria, and 40% of all *P. malariae* cases in Sarawak in 1999 occurred in the Kapit Division. Malaria

caused by *P. malariae* typically results in low parasite counts, is a benign infection that does not normally require hospitalization. However, virtually all cases in Kapit were hospitalized and some patients had high numbers of parasites in their blood. Another atypical feature was that 95% of the *P. malariae* malaria cases in Kapit occurred in adults. In order to determine whether these cases were correctly identified as *P. malariae*, blood samples were obtained from four '*P. malariae*' malaria patients at Kapit Hospital and examined by a molecular method which detects the DNA of the malaria parasites. These DNA tests showed that the patients were infected with malaria, but were negative for *P. malariae*. A variant type of *P. malariae* had been described in Asia and it was possible that our molecular test was not recognizing it because the DNA sequence was different. We, therefore, obtained the DNA sequence for one of these '*P. malariae*' patients of the target gene of our molecular detection test. Whenever a DNA sequence is obtained, it can be compared with other sequences that have been deposited in an online database called GenBank. When this was done, it caused great excitement in the laboratory because the DNA sequence was 99.6% identical with that of *P. knowlesi*, a malaria parasite typically found in long-tailed and pig-tailed macaques

Further DNA sequencing of seven other '*P. malariae*' isolates by Lee Kim Sung as part of his PhD studies, confirmed that these were also *P. knowlesi*. Since DNA sequencing is not rapid and is expensive, we developed a molecular detection test for *P. knowlesi* and conducted a study at Kapit Hospital starting in 2000. Staff at that hospital collected 208 blood samples from malaria patients and blotted them onto filter papers. These were sent to our lab in UNIMAS where they were examined with molecular detection

tests. By microscopy at Kapit Hospital, 141 (68%) were diagnosed as *P. malariae*, 42 as *P. vivax* and 25 as *P. falciparum* infections. In contrast, by our molecular tests, none of the 208 samples were identified as *P. malariae* and 120 (58%) were identified as *P. knowlesi* while the remainder were non-knowlesi and non-malariae. This was a landmark discovery since it was generally believed that malaria was not a zoonosis; an infection originating from an animal. This work, published in the leading medical journal *The Lancet*, placed Malaysia on the world map for malaria research. This was the start of a journey on which we investigated and answered many questions about knowlesi malaria.

Why did the laboratory technicians misdiagnose *P. knowlesi* as *P. malariae*?

The laboratory technologists were trained to identify the 3 major malaria parasites found in Malaysia: *P. falciparum, P. vivax* and *P. malariae*. Upon re-examination of the blood samples, it was found that they were misdiagnosing *P. knowlesi* as *P. malariae* because these two parasites looked similar under the microscope. The only way to correctly identify each species is by using the molecular detection methods, or DNA tests, that have been developed.

Had any *P. knowlesi* infections been reported before in humans?

One of the most frequently asked questions at scientific seminars after we first published our findings was whether *P. knowlesi* infections in humans had been reported before. The first detailed studies on *P. knowlesi* were conducted at the Calcutta School of Tropical Medicine and Hygiene by Dr Robert Knowles and Dr Das Gupta in the 1930s. Malaria parasites were detected

in a long-tailed macaque (*Macaca fascicularis*) (Figure 2) that was imported from Singapore. When they injected the infected blood from one Singaporean long-tailed macaque into another, it caused very mild infection and the number of parasites in the blood remained very low. However, when they injected a rhesus macaque (*Macaca mulatta*), a species normally found in India, the parasites multiplied unchecked, and the Indian rhesus macaque died. Further experiments confirmed that this parasite was benign in Singaporean long-tailed macaques but was fatal for rhesus macaques. Knowles and Das Gupta injected infected monkey blood into two humans and both got malaria. They noticed that the patients had a 24 hour fever peak. At that time, when no antibiotics were available, the only viable treatment for patients with neurosyphilis was by inducing a fever by giving the patients vivax malaria, either through an injection or by infected mosquitoes. The increased body temperature had a detrimental effect on the causative agent of syphilis and patients were cured. *P. vivax* produces a fever peak every 48 hours, so when it was found that *P. knowlesi* resulted in 24-hour fever peaks, *P. knowlesi*-infected blood was used for the treatment of patients with neurosyphilis. The largest number of patients treated this way took place in Romania, where they stopped using *P. knowlesi*-infected blood because they noted that after serial blood passages through 170 humans the parasite grew better, causing higher parasite numbers much quicker in the patients' blood.

Although it was known that humans could get knowlesi malaria by injection of infected blood in 1932, it was only over thirty years later that the first case of a human getting knowlesi malaria by a mosquito bite was reported. A US army surveyor on a five-week mapping exercise in the jungle near Temerloh in Pahang State, Peninsular Malaysia, returned to USA with a fever, and was eventually

Figure 2. Long-tailed macaque (Left; photo by Anthony Sebastian) and a rhesus macaque (Right).

diagnosed by microscopy as having *P. malariae* malaria. They took a blood sample from him and sent it to Atlanta where they were doing drug trials on *P. malariae* malaria. When they injected it into human volunteers, they noticed that their fever peaks were every 24 hours and not the expected 72 hours for *P. malariae*. Since, it was a research facility with monkeys in the animal unit, so they injected rhesus macaques with infected blood and the macaques died, confirming that the parasite was *P. knowlesi* and not *P. malariae*. This finding, that humans can get malaria from monkeys by mosquito bites in nature, was of great concern since the World Health Organisation had just launched the Malaria Eradication Programme. The American scientists that were working at the Institute for Medical Research in Kuala Lumpur returned to the same area in Pahang state where the American surveyor got infected. They collected blood samples from almost 1,200 people, pooled the samples and injected them into rhesus macaques. None of the rhesus macaques got malaria. Long-tailed macaques in the area were found to have *P. knowlesi*

parasites. The scientists concluded that monkey malaria only rarely causes human malaria and this was the generally accepted view among the scientific community.

Were monkeys in Sarawak the source of the *P. knowlesi* infections?

As there were many cases of knowlesi malaria in Kapit Hospital the next investigation by the malaria detectives was to determine whether monkeys in Kapit were the source of the human infections. Long-tailed and pig-tailed macaques infected with *P. knowlesi* had been previously described in Peninsular Malaysia and the Philippines but not in Sarawak. It was a long and labour-intensive part of the research. We had to trap wild monkeys, obtain the blood samples and then release them back into the jungle. So we constructed monkey traps in Kapit, each made in four sections so they could be transported by boat and carried long distances by foot. A trap was basically 8 feet by 8 feet square, with 8 feet high walls made out of aluminium that was smooth on the inside (Figure 3). There were also two small doors for each trap. Finding a suitable place to set them was the next challenge. We set them up near farms in the forest fringe where farmers had either seen macaques or their crops had been raided by macaques. A team of at least eight people were necessary to set up each trap and it required carrying each section of the trap for long distances from roads and river banks.

Figure 3. Monkey traps and tagging monkeys. Traps were set up near longhouses (Left) and near farms (Right). Our veterinarian, Roynston Julin, is tagging a monkey after obtaining a blood sample (Below).

For the first two to three weeks the two doors of the trap were left open and we placed local fruit such as bananas and rambutans in them. When we were certain that the monkeys were used to this free feast of fruits, we closed the doors and left a small amount of fruit on the top of the trap to lure the monkeys. They climbed

up the walls from the outside, ate the bait and then jumped down to get more fruit and were trapped. We then set a small transfer cage against one of the doors, opened it at the same time as the other door and the monkeys ran into the transfer cage. The monkeys were anaethesised and a small blood sample was taken from the femoral vein by our veterinarian on the research team, Roynston Julin. A small microchip was then inserted just under the skin at the neck of each monkey and when the anaesthetic had worn off, the monkeys were released near where they had been trapped. Whenever we trapped a monkey, we checked with a transponder reader to see if that monkey had been previously trapped. These monkeys are highly intelligent and the earliest that these monkeys re-entered the trap was six months after they were first captured!

The monkey blood samples were immediately frozen in a portable freezer (a liquid nitrogen dry shipper) and were transferred into a freezer at Kapit Hospital. They were then transported frozen to the lab in UNIMAS, where after DNA extraction, they were examined using DNA tests for *P. knowlesi* and for four other monkey malaria parasites. These new tests were developed by Paul Divis at UNIMAS to detect *P. inui, P. cynomolgi, P. coatneyi* and *P. fieldi*. Out of a total of 108 wild macaques tested, 101 (94%) had either one or more species of malaria parasites. Of the 82 long-tailed macaques and 26 pig-tailed macaques, 87% and 50% respectively had *P. knowlesi* parasites in their blood. This was the highest proportion of wild macaques with *P. knowlesi* and other malaria parasites that had been reported and remains the highest described to date.

In order to determine whether these monkeys were really the source of the infections we had to compare the *P. knowlesi* that were present in the macaques with those that were present in malaria patients at the genetic level. Dr Lee Kim Sung, our postdoctoral researcher on the project, sequenced the mitochondrial genome of *P. knowlesi* derived from monkeys and humans, while Siti Khatijah sequenced another gene called the circumsporozoite gene. When we analysed the data we found that there were clones that were shared between monkeys and humans and there were no clones that were exclusively associated with either monkeys or humans. By further analysis of the genome data we were also able to estimate that *P. knowlesi* was an ancient malaria parasite that existed before humans arrived in Southeast Asia. Monkeys were present in Asia before humans arrived and monkeys were most probably the original hosts of *P. knowlesi*.

Which mosquitoes were transmitting *P. knowlesi*?

We had shown that monkeys and humans had *P. knowlesi*, but what were the mosquito species responsible for malaria transmission? Over 3,500 types or species of mosquitoes have been described worldwide. Many of these are not blood feeders and even those that feed on blood are mainly biting nuisances and are not capable of transmitting any pathogens and causing disease. The major mosquitoes responsible for transmitting certain bacteria, viruses and parasites are *Mansonia, Aedes, Culex* and *Anopheles* (Figure 4). Of these, only certain *Anopheles* mosquitoes transmit malaria to humans because the malaria parasites can only develop and migrate from the midgut to the salivary glands in these type of mosquitoes.

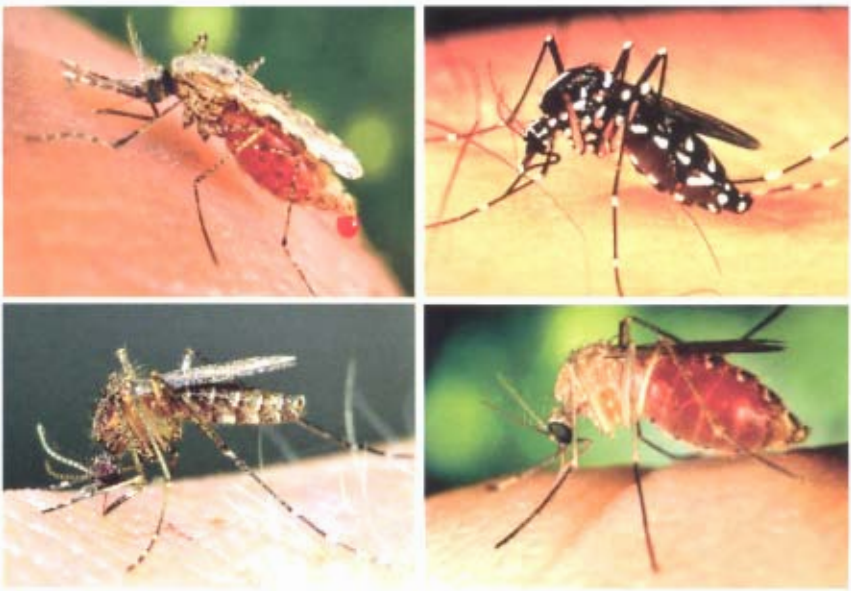

Figure 4. Major mosquitoes that transmit disease. From top left to below right: *Anopheles, Aedes, Mansonia* and *Culex*.

The mosquitoes had to be trapped, and this was another labour-intensive exercise involving a technique called 'human landing catch' (Figure 5). A team consisted of three people, and two would take turns to sit with their lower limbs below the knee exposed to mosquitoes from 6 pm until 6 am. When a mosquito landed, it was trapped in a glass vial, and the time it was trapped was noted. To determine which mosquitoes were attracted to monkeys, a pair of monkeys in a cage was placed under a large net. There was an opening which allowed the mosquitoes to enter and feed on the monkeys. Every hour, a person would enter the net, closed the opening and collect the mosquitoes that were resting after their blood meal. These 'monkey-baited traps' were set up at ground level and also on two platforms at elevations of 3 and 6 metres. This entomological study, which was part of