Advances in Malaria Research
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Advances in Malaria Research
   Edited by Deepak Gaur, Chetan E. Chitnis and Virander S. Chauhan
Advances in Malaria Research

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Dedicated to all our past and current students and group members
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Malaria is still a major affliction of humans, despite the reported decreasing trend in incidence and mortality in recent years. It is reported that the incidence has reduced from around 500 million cases by almost half, and mortality has decreased from around a million to around 700,000, although the uncertainty range is rather high. This has been achieved through public health measures to protect against mosquito bites and by quicker diagnosis followed by appropriate drug therapies. But an effective vaccine is still not available, and the drugs, including artemisinin derivatives, are prone to become less effective because the parasite develops resistance to them. With a laudable ultimate goal to eradicate malaria, the challenges remain high, and this monograph entitled “Advances in Malaria Research”, edited by Deepak Gaur, Chetan E. Chitnis and Virander S. Chauhan, is timely and captures the tremendous progress made in all facets of parasite biology.

The editors have assembled contributions from acknowledged experts to cover all facets of malaria research. Appropriately, the articles start with the process of mosquito injection of sporozoites and the development of the parasite in the liver stage, governing the pre-erythrocytic stage. This is followed by analysis of the molecular basis of erythrocyte invasion by *Plasmodium falciparum* and *Plasmodium vivax*. The next phase is obviously the transmission stage, and the next section of the book discusses rational and sustainable intervention options.

This is followed by a set of articles discussing the molecular basis of parasite metabolism. These include an analysis of the various layers of gene regulation, including epigenetic modifications and gene expression, covering all facets from transcription to post-translational modification. An understanding of the essential genes involved in erythrocyte-stage infection and adaptation to environmental changes with approaches such as conditional knockouts, followed by transcriptomic, proteomic, and metabolic pathway analysis, highlight the dynamic properties of the *Plasmodium* genome. The other molecular targets are the signal transduction pathways and transporters on the parasite membrane. At the molecular level, a detailed analysis of drug targets such as the parasite proteases and mechanisms of antimalarial drug resistance highlight the challenges involved in identifying new drug targets that can ultimately lead to drug molecules with ideal characteristics.

From the picture at the molecular level, the progression is toward an understanding of malaria pathogenesis based on the virulent features of the parasite. A major contributor to malaria pathogenesis is the host response influenced by host genetic factors and control of inflammation and acquisition of adaptive immunity. Drugs and vaccines have to be designed not only to combat falciparum malaria but also vivax malaria. *P. vivax* infection is no longer considered benign, with its unique properties for relapse long after treatment.

This monograph has been organized very logically in terms of an understanding of the challenges from the vector to the parasite and to the host in a seamless fashion, highlighting the current status in every stage of parasite development and disease manifestation. This is a volume that will be immensely useful to all malaria researchers and public health strategists.

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In the summer of 2011, Professor Angelo Azzi, the then-president of the International Union of Biochemistry and Molecular Biology (IUBMB) approached us to edit and compile a book on recent developments in malaria as part of the Wiley–IUBMB book series. This was an unexpected surprise, as we had never undertaken such an exercise before, and with great excitement we readily agreed. Angelo was eager to see a malaria book as part of IUBMB and Wiley’s association to produce high-quality books on science that would have a strong impact on both researchers in the malaria field as well as students who seek the latest information on the subject. Malaria research has witnessed a boom since the turn of the century, with the advent of new technologies and concepts. Thus, the idea of having a book that reviews recent developments in malaria research was conceived. The three of us made a proposal for the book on the same lines to Wiley and IUBMB with a potential list of contributors who we hoped would join us in our endeavor. We are eternally grateful that almost everyone whom we approached with a request to contribute to our book readily accepted our invitation. Thus, we are proud to have 17 invited chapters from top-notch malaria researchers from the field, who have all been listed in the contributors section.

However, inexperienced as we were at the start of the project, little did we know what we were getting ourselves into. As is expected in these projects, some contributors sent their chapters well within time, whereas others, also our friends, had to be pursued by all means at our disposal. We badgered them regularly at the expense of potentially losing their friendship, soldiering on continuously, and have finally successfully compiled the malaria book.
We are delighted that coincidently this book commemorates 25 years of the establishment of the International Centre for Genetic Engineering and Biotechnology (ICGEB) New Delhi component, in which malaria research was taken up right at its inception and has remained one of its major flagship programs. Scientists in the malaria group work on different aspects of malaria research, including basic biology and drug and vaccine development, with a major focus on the erythrocytic blood stages of the malaria life cycle. What is unique about the ICGEB malaria program is that there is an equal focus on both *Plasmodium falciparum* and *Plasmodium vivax* malaria.

It is only befitting our work culture in the ICGEB malaria group that the three of us came together to edit and compile the book. We wish to specially acknowledge our group members, Enna Dogra Gupta, K. Sony Reddy, Tajali Sahar, Nidhi Hans, Chaitanya, Gaurav Anand, Hina Singh, Aakansha Kalra, Anushree Bhatnagar, and Alok Pandey, who helped us at the end in the final stages of formatting and compilations. We wish to put on record that all three of us thoroughly enjoyed reading all the contributions from some of the very best malaria researchers and honestly benefitted in our own understanding of malaria research. We sincerely hope that the students and researchers will also greatly benefit from reading this book, which contains the most updated state-of-the-art information on malaria.

Deepak Gaur
Chetan E. Chitnis
Virander S. Chauhan
December 15, 2015
CHAPTER 1
Introduction: An overview of malaria and *Plasmodium*

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History

Malaria has threatened the human race for centuries. Ancient medical records from early civilizations based in India, China, and Mesopotamia have reported malaria as a disease characterized by intermittent fevers (Desowitz 1991; Cox 2002). In the fifth century BCE, the Greek physician Hippocrates classified the fever according to periodicity—*febris tertian* (every third day) and *febris quartana* (every fourth day)—and its association with splenomegaly (Cox 2002; Desowitz 1991; Pappas 2008). In the Middle Ages, the Romans coined the name *malaria* for the disease (Medieval Italian *mal*, “bad,” and *aria* “air”) because it was believed that the illness occurred due to toxic fumes and vapors arising from the marshy lands. This belief was further strengthened by the subsequent decline in malaria cases after the swamps were drained (Desowitz 1991).

It was only in 1880 that the true causative agent of the disease was identified when Alphonse Laveran (1845–1922), a military physician based in Algeria, first reported the crescent-shaped malaria parasites in the blood of a soldier suffering from intermittent fevers (Laveran 1881; Bruce-Chwatt 1981). He also observed motile filamentous structures emerging from a round spherical body, which he reported as appearing like an animal parasite. Laveran thus called this microscopic organism as *Oscillaria malariae* (Laveran 1881). Through his clinical examinations, he further observed that when he failed to detect the crescent structures there were no disease symptoms. He also observed that these microscopic organisms are cleared by quinine treatment.

Laveran’s findings were further confirmed in 1885 by Ettore Marchiafava and Amico Bignami, who, using eosin-stained blood stains, also observed amoeboid movement of the organism (Marciafava and Bignami 1894). In 1886, Camillo Golgi was able to differentiate between tertian and quartan malaria and also defined the morphological differences of parasites responsible for the two types of malaria (Golgi 1886). He reported that the parasite underwent asexual reproduction and that fever was closely associated with lysis of the red cells and release of parasites. In 1890, Grassi and Feletti named the two different species *Plasmodium vivax* and *Plasmodium malariae* (Grassi and Feletti 1890). Sakharov (1889) and Marchiafava and Celli (1890) independently identified *Plasmodium falciparum* (Grassi 1900; Cox 2002; Cox 2010). Thus by 1890, it was known that malaria was caused by a protozoan parasite that invaded and multiplied in erythrocytes. Based on their
periodic specificities and other characteristics, species specific for causing benign tertian (*Plasmodium vivax*), malignant tertian (*Plasmodium falciparum*), and quartan malaria (*Plasmodium malariae*) had been discovered (Cox 2010).

The next major question was: How does malaria transmission occur? This question was answered by the efforts of Sir Ronald Ross and Giovanni Battista Grassi. The idea that mosquitoes could transmit human disease first arose from the classical work of Sir Patrick Manson, who could be considered the father of tropical medicine and was also the mentor of Ross. In 1878, Manson was the first to demonstrate that a parasite (in this case the filarial worm) that causes human disease (elephantiasis) could infect a mosquito (Manson 1878). Ross, who was born in India, returned to India in 1895 and set about to prove the hypothesis of Laveran and Manson that mosquitoes were associated with malaria transmission.

Finally on August 20, 1897, in Secunderabad, Ross made his landmark discovery in which he observed the malaria parasite in the stomach of an *Anopheles* mosquito fed four days previously on a malaria patient, Husein Khan, who was suffering from intermittent fevers. Ross thus established the role of *Anopheles* mosquitoes in the transmission of human malaria parasites (Ross 1898). Ross was clearly on the verge of demonstrating the *Anopheles* mosquitoes to be responsible for human malaria transmission, but unfortunately he was unable to do so because at that stage he was transferred to Calcutta, a place with much less malaria.

He thus turned his attention to the avian malaria parasite, now known as *Plasmodium relictum*, which is commonly found in several bird species and which was a more convenient experimental model for malaria research. He discovered that the avian malaria parasite was transmitted by the gray (culicine) mosquito, *Culex fatigans*. Ross demonstrated malaria parasites in mosquitoes that had been fed on infected birds; the parasites developed and migrated to the mosquitoes’ salivary glands, thus allowing the mosquitoes to infect other birds during subsequent blood meals. Thus, in 1897, Sir Ronald Ross elucidated the complete sexual-stage life cycle of *Plasmodium relictum* on the gut wall *Culex fatigans* (Ross 1898).

However, the actual evidence for the transmission of human malaria by *Anopheles* mosquitoes came from Bignami and Grassi in 1898, who had access to the malarial disease prevalent near Rome and Sicily. They showed that *Anopheles claviger* mosquitoes that fed on malaria-infected patients could via their bite transmit the disease to uninfected individuals (Grassi 1899). The Italians further went on to prove that it was only the female *Anopheles* mosquito that could transmit malaria, and they comprehensively described the blood stage mosquito life cycles of *P. vivax*, *P. falciparum*, and *P. malariae* (Grassi 1900). Later in 1899, Ross, during his posting in Sierra Leone, also demonstrated the development of the three *Plasmodium* species parasites in the *Anopheles* mosquitoes (Dobson 1999). *Plasmodium ovale* was discovered much later in 1918 by John Stephens (Cox 2010; Sutherland 2010).

Thus, the mode of malaria transmission through the *Anopheles* mosquito vector had been discovered and, in a great advancement to the field, provided a major method of protecting against the disease by reducing contact with the insect vector. The huge impact of this work was recognized when in 1902 Ronald Ross was awarded the Nobel Prize and in 1907 Charles Alphonse Laveran received the prize for establishing the role of protozoans as causative agents of human disease. In 1927, Julius Wagner-Jauregg was awarded the Nobel Prize for treating neurosyphilis by infecting patients with *Plasmodium vivax* (White 2011). This treatment was abandoned because it killed 15% of the patients. The fact that mosquito control was critical led to the development of several insecticides, including DDT, for whose discovery Paul Hermann Müller was awarded the Nobel Prize in 1948.

The complete life cycle of the *Plasmodium* parasites in humans was still not fully understood, especially the liver stages of development were not known at the time, and it remained a puzzle as to where the parasites resided for the first ten days after infection when they could not be observed in the blood. Although there were some suggestions that the parasites underwent another stage of
development besides in the blood, this was clouded for about four decades under the influence of a highly prominent German scientist Fritz Schaudinn, who in 1903 claimed the direct invasion of red blood cells by the infected *P. vivax* sporozoites (Schaudinn 1903), even though these findings could not be confirmed. The first insight for the presence of an exoerythrocytic liver stage came from MacCallum’s work on avian malaria: In 1898 he observed developmental stages of *P. relictum* in the liver and spleen of infected birds (MacCallum 1898). This was finally confirmed in 1947 by Henry Shortt and Cyril Garnham, who demonstrated that a stage of multiplication in the liver preceded the blood stages in the life cycle of the primate malaria parasite, *P. cynomolgi* (Shortt and Garnham 1948a). Thereafter, Shortt and Garnham detected exoerythrocytic liver forms of three *Plasmodium* species, *P. vivax* (Shortt 1948b), *P. falciparum* (Shortt 1949), and *P. ovale* (Garnham 1954) in humans. In 1986, Krotoski discovered the dormant liver stages known as hypnozoites that are characteristic only of *P. vivax* and are responsible for the relapse of malaria (Cogswell 1992; White 2011; Markus 2012).

The life cycle of *Plasmodium*

The above-mentioned research accomplishments led to our present-day understanding of the malaria life cycle that is common for all human *Plasmodium* species (Figure 1.1). The life cycle is initiated through the bite of an infected female *Anopheles* mosquito, which during a blood meal injects the spindle-shaped invasive stages known as sporozoites into the skin, from where they travel through the blood stream to the liver. In the liver, the sporozoite travels through multiple cells, including a Kupffer cell, before finally residing in a hepatocyte. Within the hepatocyte, the single sporozoite grows and multiplies to yield around forty thousand invasive structures called merozoites. The merozoites are further released into the blood stream, where they invade red blood cells or erythrocytes. Within the erythrocyte, the parasite undergoes asexual reproduction known as schizogony, allowing a single parasite to produce 16 to 32 daughter merozoites, which, following egress, are released into the blood stream, leading to another cycle of erythrocyte invasion and growth.

It is the blood stages of the life cycle that are responsible for the clinical symptoms and pathology associated with the disease. The preceding exoerythrocytic liver stage does not produce any clinical symptoms, and in *P. vivax* infections, the hypnozoites could persist for decades without any symptoms. During the blood-stage life cycle, some parasites fail to progress and divide for reasons that still remain poorly understood. Instead, these parasites differentiate into gametocytes, which appear as crescent-shaped structures and were first observed by Laveran. During a blood meal, the male and female gametocytes are taken up by the *Anopheles* mosquito, the invertebrate vector host in which the sexual stage of the parasite’s life cycle is completed. The male gametocyte forms motile microgametes that undergo exflagellation and swim to the macrogamete formed from the female gametocyte. The microgamete fertilizes the macrogamete resulting in the formation of the motile zygote, known as the ookinete, which traverses the epithelial lining of the midgut and finally resides in a thick-walled structure known as the oocyst under the mosquito’s outer gut lining. Within the oocyst, several sporozoites are formed through asexual multiplication and make their way to the mosquito’s salivary gland. It is these sporozoites that get injected in to the human host through the bite of the mosquito, thus completing the life cycle that progresses from there.

**A significant milestone in malaria research: Adaptation of *Plasmodium* to laboratory culture**

It has been more than a century since Ross’s landmark discovery, and during this ensuing period, the first fifty years of research on malaria parasites were focused on describing the parasite, identifying it as the causative agent of malaria, understanding its mode of transmission, and uncovering the different stages of its complex life cycle. A major obstacle during this period was the difficulty in culturing the parasite *in vitro* in the laboratory, due to which primary experiments were performed with primate models such as *Aotus* monkeys and even human volunteers. Initial attempts to culture *Plasmodium* parasites failed because the cultures were always short term, in which the parasite numbers were constantly decreasing till they completely died.

The continuous culture of *P. falciparum* was successfully established by William Trager and James Jensen in 1976. The novelty of their work was to try to culture the *P. falciparum* clone FVO obtained from an *Aotus* monkey under low oxygen concentrations (5% O₂/7% CO₂/88% N₂) with commercial RPMI 1640 medium in flow vials with a settled layer of human red cells (Trager and Jensen 1976). In these conditions, Trager and Jensen were able to culture the parasites for 24 days with timely addition of fresh red cells every 2 to 3 days. Another innovative development from them was to employ an old microbiological candle-jar method that was historically used for anaerobiosis to generate a CO₂-rich atmosphere. This simple methodology made it much easier to culture malaria parasites even in remote settings that did not have access to mixed gases (Trager and Jensen 1976).

Whereas Trager and Jensen were the first to report the continuous culture of *P. falciparum* in their *Science* paper published in August 1976, another group, led by J. David Haynes, also demonstrated the same findings, which were published the same year in the journal *Nature* in October (Haynes 1976). Haynes demonstrated that in addition to human red cells, chimpanzee red cells also supported *P. falciparum* growth but red cells from rhesus monkeys and guinea pigs did not (Haynes 1976). The present generation of malaria researchers might find it difficult to envisage a time when *P. falciparum* culture was not possible because it is such an important and integral part of current daily efforts in malaria research. Thus, it is difficult to gauge the enormous impact of continuous *P. falciparum* culture cultivation on malaria research across the world.

Probably no other single development has had such a profound and unparalleled influence on malaria research as observed with the laboratory culture of malaria parasites. It completely
revolutionized malaria research and enabled studies on different aspects of malaria that are being pursued with great vigor even today in fields include biochemistry and molecular and cellular biology of the parasite; host–parasite interactions especially, with human red cells; immunology and vaccine development; drug development and resistance; pathogenesis; gametocytogenesis and mosquito transmission; and genetics of the parasite and red cell susceptibility. Basically, malaria parasite culture brought accessibility to scientists who otherwise did not have access to clinical malaria cohorts and thus allowed an astounding expansion in malaria research. Overall, the successful \textit{in vitro} culture of \textit{P. falciparum} remains the basis of present-day malaria research.

\textbf{The advent of present-day technologies and their applications in malaria research}

Malaria research has benefited immensely with the onset of new innovative technologies, high-throughput platforms, and novel biochemical and molecular approaches, which have led to a boom in major discoveries that have unraveled the intricacies of the malaria parasite at a molecular level. The past couple of decades have seen the growth of -omics technologies that have been vigorously applied to malaria parasites. First was genomics, which with the help of advanced sequencing techniques and instrumentation allowed the sequencing of various pathogens including \textit{Plasmodium} species. The publication of the complete sequence of the \textit{P. falciparum} genome in 2002 (Gardner 2002) was a landmark accomplishment that was followed by the genomes of \textit{P. vivax} (Carlton 2008), \textit{P. knowlesi} (Pain 2008), \textit{P. cynomolgi} (Tachibana 2012), \textit{P. yoelii} (Carlton 2002), \textit{P. berghei} (Hall 2005), and that of the \textit{Anopheles} mosquito vector (Holt 2002). The genome provided a complete map of all possible genes and noncoding regions of the parasite that greatly benefited molecular studies.

This was followed by transcriptomics, which provided the description of the transcriptome of \textit{P. falciparum} (Le Roch 2003; Bozdech 2003) and later \textit{P. vivax} (Bozdech 2008) through the application of high-throughput microarrays. The transcriptome provided a timeline for the expression of gene transcripts during the complete 48-hour intraerythrocytic life cycle. These studies provided a blueprint that showed how regulation of gene expression in the malaria parasite was so tightly controlled and sparked further research to identify transcription factors and understand gene regulation in \textit{Plasmodium}.

The transcriptome was complemented at the level of both protein translation and metabolites by proteomics and metabolomics (Johnson 2004; Hall 2005; malERA Consultative Group 2011). Advances in proteomics and metabolomics were set in motion with the advent of new-generation, highly sensitive mass spectrometry that could detect molecules and their modifications with great precision and accuracy. These data, together with development of methods for genetic manipulation of malaria parasites, have made it possible to probe the function of individual genes to understand the biology of these pathogenic organisms.

Research on malaria is a wide field that encompasses multiple disciplines. These include clinical research, epidemiology, and translational research toward development of new drugs and vaccines at one end and reductionist approaches to study the molecular and cell biology of malaria parasites at the other end of the spectrum. Often, to address problems in malaria effectively, there is a need to combine approaches such as the application of molecular approaches to studies in the field. This book covers the whole range of such topics to provide a snapshot of our current understanding of malaria in all its diverse aspects. It provides an update on our understanding of the biology of malaria parasites at different life cycle stages, their interaction with the host and vector, the epidemiology of malaria, and the progress made in the development of novel prophylactic and therapeutic
strategies against malaria. While remarkable progress has been made, there are still significant gaps in our knowledge. This book points out these gaps and suggests new directions to address these unsolved problems. There is a need to continue our efforts to improve our understanding of malaria, which is key to developing novel strategies to control, eliminate, and, hopefully, eradicate malaria in the future.

Bibliography


Introduction: An overview of malaria and *Plasmodium*


