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Malaria research in the post-genomic era

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Abstract

In many pathogens genome-dependent methods can partially substitute for powerful forward genetic methods that have advanced model organism research for decades. In 2002 the genome sequence of the parasite causing the most severe type of human malaria, *Plasmodium falciparum*, was completed, eliminating many of the barriers to performing state-of-the-art molecular biological research on malaria parasites, and beginning a renaissance of sorts. Although new, licensed therapies may not yet have resulted from genome-dependent experiments, they have produced a wealth of new illuminating observations about the basic biology of malaria parasites, and it is likely that these will eventually lead to new, rationale, therapeutic approaches. This review will focus on the basic research discoveries that have depended in part, on the availability of genome sequence.

Over the millennia malaria has shaped the human genome, encouraging otherwise disadvantageous alleles to persist in the at risk population as protection from this deadly disease. In addition, it has negatively affected human society by decreasing productivity and economic growth, increasing poverty ¹. Although eradicated in some parts of the world at present 40% of the world's population lives in malarious regions ². On the other hand, renewed interest in malaria prevention, the use of impregnated bednets and more efficacious drugs may have reduced the number of cases in the last several years and there have been sporadic reports that some areas are seeing few malaria cases ³.

The estimated 515 million cases of human malaria ² each year are generally caused by four species, including *Plasmodium falciparum*, *P. vivax*, *P. ovale*, and *P. malariae* and are transmitted by the bites of female anopheline mosquitoes (Figure 1). *P. falciparum* malaria has the greatest toll on human health, primarily in children under five. In addition to the characteristic fever and anaemia, neurological involvement may lead to an unarousable coma and in some cases death. If a child survives severe malaria his or her ability to learn may be impaired ⁴ reducing his or her lifelong potential. *P. falciparum* is found at high levels in Africa while in Asia and the Americas it is more common to find *P. vivax* malaria, which produces fewer fatalities but which nevertheless can be severe ⁵. While *P. falciparum* malaria is associated with a recent exposure to an infected mosquito, *P. vivax* and *P. ovale* parasites enter human hosts via the bite of an infected mosquito and then may remain dormant in the liver as the hypnozoite forms for months or years, producing no outward manifestations of disease but resulting in relapses months or years after an individual has left a malaria endemic region. This is likely an adaptation to a temperate or subtropical climate where mosquitoes may not be present throughout the year.

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Malaria control

Most malaria blood infections can be effectively treated with existing drugs although resistance is a serious problem (reviewed in ⁶). Chloroquine and antifolate drugs such as the sulfadoxine/pyrimethamine combination are safe and are still used in some regions because of their low cost. However, their continued use poses considerable risk due to widespread parasite resistance to these drugs as well as to others. At present the World Health Organization is recommending the use of drugs that contain artemisinins, antiprotozoal endoperoxides from sweet wormwood (*Artemisia annua*) in areas such as sub-Saharan Africa where resistance is widespread. While artemisinin-based drugs have many attractive features including potency and rapid action, they are rapidly eliminated and have a complex chemical structure, which has thwarted researchers' attempts to synthesize them inexpensively in the laboratory. On the other hand, compounds with similar pharmacophores have been synthesized ⁷ and it may even be possible to engineer yeast to produce such compounds ⁸. Currently the drug of last resort, parasite resistance to artemisinin-based drugs has been observed in the rodent-models for malaria ⁹, and there are a few potential cases of treatment failure in patients ¹⁰. While these reports have not yet been confirmed in cultured parasites it seems likely that full-blown resistance to artemisinins will eventually be observed, emphasizing the need for continued drug discovery research. Primaquine remains the only licensed drug that can provide a radical cure of *P. vivax* but this drug, in addition to its other liabilities, produces hemolytic anaemia in individuals with glucose-6-phosphate deficiency.

Although drugs currently work well against malaria, a vaccine that targets *P. vivax* or *P. falciparum* would likely reduce much of the poverty associated with malaria and greatly assist in complete malaria eradication. Recently encouraging results have been obtained for a vaccine, named "RTS,S", from GlaxoSmithKline Biologicals that is targeted for licensure in 2011. The vaccine contains a recombinant malaria protein fused to the surface of a Hepatitis B protein and is based on the *P. falciparum* circumsporozoite protein (CSP), an abundant surface protein associated with the pre-erythrocytic phases of parasite development. In limited clinical trials the vaccine has been shown to reduce the number of severe cases of malaria ¹¹ and delay the time to the first clinical episode. Unfortunately, the same studies also showed that children still developed malaria, raising questions about efficacy and drawing comparisons to recent unsuccessful and costly attempts to develop an HIV vaccine. Despite this, the data show that RTS,S is likely to decrease malaria severity and morbidity and were RTS,S to fail there are still more than 40 subunit vaccines in development and 16 in clinical trials (www.malariavaccine.org or reviewed in ¹²).

It has been known for a number of years that if sporozoites, the parasite form found in mosquito salivary glands, are attenuated and given as a vaccine, protection superior to that observed with a natural infection is acquired. This has led to the development of a whole organism vaccine ¹³. While this vaccine model may be less practical than single subunit vaccines due to the cost, distribution challenges, and problems with achieving consistent good manufacturing protocols, it still remains the most effective method for inducing protective immunity and efforts are being made to overcome these obstacles. While the fact that we still don't have a fully protective and licensed malaria vaccine despite decades of effort may be disheartening one must keep in mind that the development of drugs and vaccines, which are just now entering large-scale clinical trials, was initiated decades ago in a very different era for malaria research. In this period researchers were limited by their ability to work with malaria parasites in the laboratory, and thus vaccine research efforts were mostly focused on a relatively small number of very abundant proteins that could be easily studied.

Malaria Genomes

In 2002 the complete genome sequence of *P. falciparum* was published¹⁴, along with a partial sequence of *P. yoelii yoelii*, a rodent parasite often used in vaccine research¹⁵. In 2005 partial sequences of several other rodent parasites were published¹⁶ and in this issue, the genome sequences of *P. vivax* and *P. knowlesi*, primarily a monkey parasite, are described^{17,18}. In addition, the human genome sequence as well as several races (PEST, M and S) of *Anopheles gambiae*, the species of mosquito that transmits *P. falciparum* malaria have been determined (<http://genome.wustl.edu/> and <http://www.tigr.org/>) and in some cases published¹⁹. Genome sequences promised a new paradigm in which drug targets might be plucked from the genome²⁰ and there would be an acceleration of preclinical candidates into the drug and vaccine pipelines. While it may be too early to determine whether public health will be improved, knowledge of the genome has dramatically enhanced the pace of basic research on parasite biology, which may eventually lead to the production of new drugs and vaccines. Here we review how the genome has enlightened malaria research and opened new avenues of research that may eventually lead to new cures.

The *Plasmodium* genomes^{14,15,16,17,18}, are estimated to contain 23-27 million bases, 14 chromosomes and ~5500 genes, including many members of multigene families likely to be associated with immune evasion and antigenic variation. The sequenced *Plasmodium* genomes are also all rich in low complexity regions, which may assist in generating antigenic diversity through mitotic recombination. Furthermore, the genomes of *Plasmodium spp.* generally have a high AT-content. However, the genome of *P. falciparum* is exceptionally AT rich (79.6%) throughout the genome, while only portions of the genome of *P. vivax* are (overall AT content of 67.7%) indicating that the extreme AT content in itself has likely not too much to do with the disease. Nevertheless, it is possible that genes that are exceptionally AT-rich in both species may be more recombinogenic and more likely to be involved in immune evasion. Indeed, associations between AT-richness and recombination frequency have been observed in many species. There are differences. For example, in *P. falciparum* many of the multigene families involved in immune evasion are for the most part located near the ends of chromosome and are often transcriptionally silent, suggesting possible mechanisms by which genetic diversity could be generated by nonhomologous recombination across chromosomes and epigenetic regulation could be established. However, in *P. knowlesi* members of multigene families are scattered across the chromosomes and are not strictly subtelomeric¹⁸. Despite the differences 77% of the proteins are conserved across the different species.

Although parasite genome sequences are available many of the genes identified do not have homologs in commonly studied model organisms and often lack a clear cellular function¹⁴. Even with the recently completed *P. vivax* study almost half of the predicted genes still lack characterized orthologs in other systems. Considering the impact that malaria parasites have had on human health it seems remarkable that we know as little as we do. For example, we do not know the basis for sex determination in malaria parasites or exactly how parasites become committed to sexual development. Little is known about the liver stages, how the parasites home to the liver but then transverse some cells, but form parasitophorous vacuoles in others. The situation is particularly acute in *P. vivax*, which cannot be cultured. In fact there is debate as to whether the hypnozoite, the dormant parasite liver form involved in relapse in *P. vivax* malaria, is a distinct stage of merely an early exoerythrocytic form whose development has been arrested. Recently it was shown that parasite metabolism inside of a human may differ from parasite metabolism in laboratory culture, again highlighting how little is known about the role of the parasite genes during active patient infections²¹. While research into such questions may be regarded as peripheral to the central objective of finding better drugs and vaccines, further investigation into basic biology is likely to assist translational research in serendipitous ways.

Functional studies of malaria genomes

The goal of functional genomics is to determine what the different genes encoded by genome are likely to be doing for the parasites, often using high-throughput tools such as microarrays or mass-spectrometers. Transcriptional and proteomic analyses of the complement of genes encoded by the genome at different lifecycle stages can serve as a form of “virtual genetics” in which regulons of co-transcribed genes can be defined by virtue of their expression pattern across many different conditions. Thus if a gene shows a large induction during early liver stage development, there is a good chance that this is the time when its protein product will be required^{22,23}. These data can then be used to predict possible functions for parasite genes and to overcome the lack of powerful forward genetic methods that have advanced knowledge in so many other organisms. Comprehensive expression analyses of different parasite lifecycle stages have been performed for a number of malaria parasite species^{16,21,23,24,25}. Because these data are only predictions other high-throughput methods for collecting genome-wide data can assist in determining which of the genes revealed in a gene expression experiment are likely to be involved in a process of interest. Two-hybrid mapping²⁶, and proteomics^{16,27,28} produce complementary data. Comprehensive analysis of the parasite proteome has identified some of the genes contributing to the phenotypic differences between parasites of different sexes²⁹. Genome database websites such PlasmoDB (<http://www.plasmodb.org>) have served to disseminate the data from such studies and have assisted the research activities of everyone interested in malaria. New genetic tools^{30,31} have been introduced which assist those interested in testing these predictions and may eventually be used in large-scale knockout or mutagenesis projects. Genome-scale protein crystallization projects are also underway which should also help the pace of drug development^{32,33}.

One tidbit that has come from comprehensive proteomic and transcriptional profiling is that translational control is likely to play a significant role in gene regulation during development. Although experiments have generally shown that there is a good correlation between transcript and protein abundance, there are notable discrepancies. In a number of cases genes appear to be transcribed but then not translated until the organism has made the rapid transition between warm and cold-blooded hosts. Most notably, transcripts needed for gamete formation in the mosquito are produced in gametocytes in the mammalian host, but only translated after the transition, initiated by an increase in xanthurenic acid and calcium-dependent signalling cascade³⁴, has been made^{16,35}. Specific genes involved in translational repression have been characterized³⁶. While this translational silencing clearly plays a role in gene regulation when the parasite moves from the human to the mosquito, it seems likely this is also occurring in the sporozoites, which inhabit the salivary glands of mosquitoes. The sporozoite form must also be prepared for a rapid and unexpected transition to a warm-blooded organism. Indeed, the Liver Stage-Associated Protein-2 is not detected until two days after the parasite invades the liver³⁷, even though its cognate transcript is one of the most abundant in the sporozoite salivary-gland transcriptome²⁴. Accordingly, it was noted that the malaria parasite genome seems to encode many genes involved in translational regulation, such as helicases and RNA binding proteins, but relatively few transcription factors¹⁴ and many of these are transcribed in gametocytes and sporozoites. Many noncoding RNAs are transcribed by the parasite genome and some of these may play interesting roles in controlling developmental processes³⁸.

Expression profiling has also revealed groups of genes that are likely to play a role in the parasite's interaction with the mosquito²³ and which could be candidates for transmission blocking vaccines. Transmission blocking vaccines (reviewed in³⁹), which would be directed toward proteins associated with the sexual phases of development (gametocytes or gametes), would not protect from the clinical manifestations of malaria but would altruistically prevent an infected individual from passing the disease on to the next person, potentially assisting in eradication. Functional genomic methods and mosquito genome sequence have been used to

discover genes involved immunity⁴⁰ and in host parasite interactions. Work has shown that some of these genes are critical for parasite survival or are associated with the mosquito's ability to clear parasites⁴¹ suggesting new vector-based control strategies⁴². While there are of course logistical, environmental and ethical difficulties associated with releasing recombinant mosquitoes that are unable to transmit malaria, the idea remains appealing.

Genome-wide analysis of antigenic variation

Creating catalogues of genes potentially involved in parasite processes or understanding parasite development may seem arcane and unrelated to human health, however, genome-wide transcription studies can shed light on how parasites evade the host immune system. It is generally accepted that transcription of genes known to be involved in antigenic variation can switch among different members of the family and that this may contribute to immune evasion and pathogenicity⁴³. Expression analysis and genome sequence has permitted the transcription of each of the 60 var genes, which encode versions of the variant surface antigen, PfEMP1, to be monitored, ultimately providing clues as to how antigenic variation in parasites may be regulated^{44,45,46}. Genome-wide transcriptional and other studies have indicated that transcriptional switching may not be confined to var genes, encoding PfEMP1s, and involve a large number of other different gene families such as those involved in parasite invasion^{47,48}.

Importantly, these expression data have also helped in the development of innovative vaccination strategies. For example, analysis of high-throughput *P. falciparum* proteomic data²⁷ revealed one exceptionally abundant sporozoite protein⁴⁹, subsequently named CelTos in *P. berghei*⁵⁰, which was more immunogenic than some of the historical antigens such as CSP. Antigens derived from this protein are found in an experimental vaccine that is entering clinical trials⁵¹. It has also been known that vaccination with radiation-attenuated parasites can lead to better protective immunity than can be achieved with a natural infection. Expression analysis has led to the identification of parasite genes, which are specifically transcribed while the parasite resides in the mosquito salivary gland⁵². Disruption of these genes^{53,54}, or ones with similar patterns of transcription⁵⁵ has led to genetically attenuated parasites which are unable to successfully colonize the vertebrate host but which never-the-less induce a protective immune response.

Genetic diversity in malaria parasites

The identity and geographic distribution of alleles that contribute to drug resistance forms the foundation for public policy on antimalarial drugs. Genome-dependent methods have the potential to change how genes involved in drug resistance are discovered. In the past genes involved in drug resistance were identified through mapping studies⁵⁶, or through candidate gene approaches where alleles predicted to have a role in drug resistance in a different organism were correlated with resistance. Because microsatellite typing has previously revealed that haplotypes that surround the alleles involved in chloroquine resistance⁵⁷ and antifolate resistance⁵⁸ are statistically overrepresented in parasite populations, there has been interest in collecting SNP data in order to create a "haplotype" map that can be used to identify new regions of the genome in disequilibrium in different drug-selected parasite populations^{59, 60,61}. Genome-dependent methods have also revealed new candidate genes that may be involved in drug resistance. Comparative genome hybridization with high-density oligonucleotide arrays show different strains of *P. falciparum* contain numerous copy number variations (CNVs) that may be associated with drug resistance. For example, most strains carry diverse CNVs centered around the multidrug resistance transporter as well as around the first gene in the folate pathway, GTP cyclohydrolase⁶². This latter gene amplification event may be a signature of widespread antifolate drug use.

Studies of genetic variation^{59,60,61,62,63} have provided clues as to why a universally effective single subunit malaria vaccine may be difficult to develop. Clearly there are vastly different rates of variability in different parasite gene classes, with many genes involved in “housekeeping” functions, such as ribosomal proteins, DNA replication enzymes, or components of the cytoskeleton exhibiting very low levels of variability as one compares the genome of different *P. falciparum* isolates from different continents, suggesting a recent population bottleneck or a selective sweep⁶⁴. In contrast, many genes which are members of the multigene families, or which are membrane proteins show very high levels of variability⁶⁵ possibly indicating a basal evolution rate that is much higher than for housekeeping genes, occurring even during standard laboratory culture. These data also show high levels of allelic diversity in many of the genes that correspond to antigens used in vaccine trials, including CSP, the Apical Membrane Antigen 1 and the Merozoite Surface Protein 1. In some cases the high level of variability in these proteins may explain allele-specific immunity that is sometimes observed⁶⁶. These diversity data suggest it may be difficult to develop a single subunit vaccine that can provide universal protection but also point to new vaccine candidates that show imprints of host immune selection.

From the genome to cell biology

One of the more exciting recent developments that has resulted from the genome sequencing efforts has been the observation that many of the proteins that eventually find their way out of parasite and onto the surface of infected red cells carry a conserved protein export motif, called a VTS or Pexel motif^{67,68}. After a cell is infected, malaria parasites form a parasitophorous vacuole in the host cell and soon afterwards a tubovesicular network in the host cells to promote protein trafficking. Trafficking is likely to go in both directions with both import of nutrients and export of proteins involved in immune evasion occurring. Remarkably, the Pexel/VTS motif is also found in exported proteins from the plant pathogen, *Phytophthora infestans*, the organism responsible for the blight that caused the Irish potato famine⁶⁹. Here the motif is attached to small proteins introduced into the plant cytoplasm where they interfere with the plant defence systems by preventing protease activation and subsequent apoptosis^{70,71}.

Many of the 400 or so *P. falciparum* proteins that contain the Pexel/VTS motif appear to be involved in creating knobby structures on the surface of the infected red cell that are associated cytoadherence and antigenic variation, however, the motif is also found in proteins of parasites that do not cytoadhere, such as *P. vivax*⁷². In addition, while many are transcribed at the time that the tubovesicular network is established during development in the erythrocyte, a few appear to be transcribed in the pre-erythrocytic phase, at about the same time that proteins required for early liver stage development are transcribed⁷². One such protein is CSP, the target of the RTS,S vaccine, which bears two Pexel/VTS motifs⁷³. CSP has attracted attention over the years because it is likely the most abundant protein synthesized in the infectious sporozoite stage of the parasite's lifecycle. The protein is immunogenic, functioning potentially as the immunodominant antigen in the irradiated sporozoite vaccine⁷⁴. Although it has an essential role in the mosquito phases⁷⁵ of parasite development its exact cellular role has been a matter of some debate. Recently the Pexel/VTS motif from CSP was attached to reporter proteins, where it directed the reporter protein into the cytoplasm of cells⁷³, raising the possibility that CSP might have some role in host pathogen interactions. Remarkably, transient overexpression of CSP in HeLa cells appears to have a substantial effect on host transcription, downregulating many genes involved in immune signaling and upregulating other genes involved in cell adhesion and possibly apoptosis⁷³. It has been noted that parasites can grow for many days in the liver without triggering apoptosis or an inflammatory response and once they have matured in the hepatocytes and are ready to be released, the infected hepatocytes round up and lose their ability to adhere⁷⁶. Active immune modulation is known to occur in other parasitic infections, such as schistosomiasis and is likely to be a signature of a successful

pathogen. Indeed, a secreted protein kinase affecting gene expression has recently been identified as a major virulence factor in *Toxoplasma gondii*, a closely related apicomplexan parasite^{77,78}.

The genome and drug discovery

The availability of genome sequence has also coincided with in a renaissance in malaria drug discovery as malaria research has become more tractable and more funding has been directed toward drug development through the establishment of public private partnerships. When the genome sequences first began to appear, researchers noted several pathways that appeared to be present in parasites but absent in higher eukaryotes²⁰. These included the type II fatty acid biosynthesis pathway that is associated with the apicoplast, a unique organelle found in apicomplexan parasites. While novel, parasite-specific drug targets are still of substantial interest, recent drug discovery campaigns may be shifting from the single-enzyme screening approaches to cell-based methods where one can test for inhibition of all essential proteins simultaneously^{79,80,81}. Cell-based methods have been less attractive in the past because of the difficulties associated finding a compound's mechanism of action. The availability of genome-dependent methods such as tiling microarrays or deep full genome sequencing that can pick up new mutations that have been introduced in the laboratory as a consequence of drug pressure, and sensitive proteomic methods that can be used in affinity chromatography experiments, as well as new robots and high content imaging systems may also make the daunting task of discovering what compounds are doing in the cell less challenging.

Despite the advances there is still much work ahead. For example, the RTS,S and irradiated sporozoite vaccines are both imperfect and many feel that other approaches should be examined in parallel, especially as RTS,S is only partially efficacious. There are also logistical hurdles associated with distributing either an attenuated or killed organism vaccine. Testing vaccines remains extremely expensive because there is no way to tell whether a vaccine is effective except through the use of human subjects. There are hurdles in the drug development front because it is likely that most of the compounds that have shown the ability kill parasites in the laboratory setting will not be modifiable into something that could be feasibly and safely delivered to patients in Africa. In addition to problems with delivering drugs, educating local physicians and accessing at risk populations, there are inadequate methods available for surveillance and to even determine whether roll back malaria campaigns are having an effect. Despite this, during this renaissance in malaria research optimism has reached a point where some are calling for new campaigns to completely eliminate malaria. If basic research continues to be a priority and if support is sustained, new drugs and effective vaccines are likely to be developed, and this could make the goal of global malaria eradication achievable.

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Figure 1.

Diagram of the malaria parasite's lifecycle. Malaria is transmitted by the bite of a mosquito in which hundreds of sporozoites are released into the vertebrate host's bloodstream. The parasites eventually migrate to the liver, transversing some cell types such as Kupfer cells but forming a parasitophorous vacuoles in hepatocyte. At this stage they can either remain dormant as a hypnozoite form (*P. vivax* or *P. ovale*), or initiate development that results in the production of thousands of merozoites. The parasites then induce detachment of the infected hepatocyte, allowing it to migrate to the liver sinusoid where budding of parasite filled vesicles called merosomes⁷⁶ occurs. The new merozoites quickly invade erythrocytes where they replicate, sometime synchronously, in a cycle that may correspond to the cycle of fever and chills in malaria. In response to a cue that is not well understood some parasites differentiate into male and female gametocytes, which are forms taken up by the mosquito, and can live quiescently in the bloodstream for weeks. Once they enter the mosquito in the bloodmeal they rapidly transition into activated male and female gametes. The motile and short-lived diploid parasite form, the ookinete, migrates out of the bloodmeal, across the peritrophic matrix to the midgut wall where an oocyst is formed. After a meiotic reduction in chromosome number thousands of sporozoites are formed within the oocyst. Eventually the oocyst ruptures and the sporozoites migrate to the salivary gland where they await transfer to the vertebrate host.

Figure 2.

Export pathways shared by eukaryotic pathogens. A. In erythrocytic stages of the parasite exports proteins containing a Pexel/VTS motif across the parasitophorous vacuole into cytoplasm of the infected erythrocyte^{67,68}. Some of these proteins eventually reach the surface of the infected erythrocyte where they have a role in antigenic variation and immune evasion. B. In plant pathogens a similar pathway is used with the effector proteins often interfering with plant innate immune defences⁸². C. In the liver stages of a malaria infection, CSP, and potentially other proteins, are released into the hepatocyte cytoplasm. One model is that these proteins may interfere with import of nuclear factor KB⁷³, a protein needed for activation of human innate immune responses.