

Blue skies or stormy weather: what lies ahead for malaria research?

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During the past ten years, our understanding of many aspects of the biology of malaria parasites has increased dramatically. In particular, the complete genome sequences of *Plasmodium falciparum* and *Plasmodium yoelii*, the availability of transcriptome and proteome profiles, and the establishment of transfection techniques for asexual-stage malaria parasites all represent major achievements from the past decade. Now that we are truly in the post-genomic phase of biological enquiry, this article highlights some of the opportunities and challenges that lie ahead, and speculates on what we should expect to achieve in the future.

A review of the malaria literature from the 1970s and early 1980s reveals that malaria infected 200 million people and killed around one million every year, and that the development of an effective vaccine would take around ten years [1]. Some 25 years on, building on the outcomes of intensive research funded by, among others, the National Institutes of Health, the Rockefeller Foundation, the John D. and Catherine T. MacArthur Foundation, the Burroughs Wellcome Fund, the World Health Organization, the National Health and Medical Research Council of Australia, the Medical Research Council of the UK and the Wellcome Trust, what is the situation? Malaria now infects 500 million and kills up to three million people annually, and an effective vaccine is not even 'just around the corner' [2,3]. Have we accomplished anything? We know more about the biology of the parasite and its interaction with the host, but we cannot yet claim that our research efforts have delivered substantial improvements to the health and well-being of almost half of the world's population, whose daily lives are curtailed by malaria infection. We do know that the problem of malaria control through vaccines and drugs is much more difficult than we had first thought. It is not only a research problem, but also a problem of financing, market forces, political will and logistics, whose solutions will need to come from many sectors. The slow dissemination of insecticide-treated bednets in various malaria-endemic areas demonstrates that even when a clearly beneficial intervention is identified, it might be some time before it is fully deployed. There is an additional financial barrier in the development of lead compounds, in addition to assessing the efficacy of potential new drugs and vaccines. In this

regard, we must laud the commitment of private support such as that provided by the Malaria Vaccine Initiative (<http://www.malariavaccine.org>) and the Gates Foundation, and the public-private partnerships developed by the Medicines for Malaria Venture (<http://www.mmv.org>), which allow such progress.

Genomes and beyond

There is probably not any human activity more certain of failure than trying to predict the future pace and direction of scientific research. However, the likely expectation of failure has never deterred us. If it did, we would not work in malaria research. So, what will the next four years bring? The paradigm of whole-genome research will dominate the near future. The completion and annotation of the genomes for *Plasmodium falciparum* [4] and its *Anopheles gambiae* mosquito vector [5] sets us a formidable challenge to determine the function of the multitude of novel gene products, and to establish precisely how they interact in space and time to co-ordinate important aspects of transmission, invasion, growth, host red blood cell modification and the pathogenesis of this devastating parasite. Complexity is increased by the availability of the genome sequence of the rodent malaria parasite, *Plasmodium yoelii* [6], and the impending arrival of several others including *Plasmodium vivax* [7]. However, there is one benefit in the refinement of gene prediction and a rapid identification of genes specific to parasites with particular host ranges and pathogenic capabilities. Investigation of tractable experimental systems such as yeast, *Escherichia coli* and *Caenorhabditis elegans* has provided several precedents as to how three-dimensional protein structure, mass mutagenesis screens, transcription patterns, interaction maps and comparative genomics can yield important insights into protein function. How applicable is this type of whole-genome scanning to malaria? The answer is, at present, only partly applicable. We already have initial transcriptome and proteome profiles for *P. falciparum* [8], and a smaller dataset for *P. yoelii* [9,10] with other species to follow. These will be refined further with more gene-sequences being added to the arrays when the *P. falciparum* genome is finally finished and the orphan genes identified. However, even the initial proteome results show the need for improved methods of cell fractionation, particularly for organelles such as plastids, micronemes, rhoptries, Maurer's clefts and the membranes of parasitized red blood cells (pRBC).

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Mass screening and other methods

The application of many mass-screening methods is seriously hampered by the inefficiency of transfection of asexual-stage malaria parasites [11,12]. This is an area in urgent need of development, and a systematic survey of available parasite lines to identify those that show a high efficiency of transfection and a low rate of gametocytogenesis would be clearly worthwhile. The absence of reliable transformation vectors that are stably maintained as episomes or that recombine into the genome at high efficiency is still a serious impediment to the development of a mutagenesis screen, much less the absence of a transposon system that functions in malaria. Lack of efficiency also hampers several methods that examine protein–protein interactions in a systematic manner, such as the affinity-tag method [13]. Hopefully, the promising results of Meissner *et al.* [14] on a tetracycline-dependent transactivator system that allows inducible gene expression in malaria will be rapidly translated into widely available methods for analysis of gene function.

Solving these problems would allow systematic generation of knockouts to elucidate gene function. However, there is a more fundamental problem – the paucity of phenotypic assays in malaria. Even relatively crude parameters such as the growth rate *in vitro* lack a generally agreed standard method for determination, and the capacity of parasites to grow under stressed conditions remains inadequately studied. There is a need for quantitative assays to study structural and morphological aspects of the parasite. For example, we cannot accurately quantify the number of micronemes per parasite, or determine the exact number of knobs or Maurer's clefts in a pRBC. We cannot accurately quantify the number of copies of specific parasite proteins that are secreted or exported to particular locations in the host red blood cell, measure secondary metabolic events, quantify cell signaling, or determine the extent of phosphorylation of parasite and host proteins [15,16]. Quantifying these parameters by relatively high-throughput screening procedures, and identifying new and possibly more-subtle phenotypes will require a major investment in cell biology, biochemistry, biophysics, imaging and model systems. The use of new biophysical techniques for quantifying the effect on pRBC of protein–protein interactions at the micro- or nano-scale are also worthy of further exploration. This will assist in the assessment of altered cellular mechanical properties that contribute to the pathophysiology of the disease (for a recent review, see Ref. [17]). Moving to *in vitro* systems that measure protein function also run into a major technical difficulty in producing recombinant protein. We have been able to produce full-length protein for ~20–30 genes over the past 20 years, and a huge increase in effort will be required to express an additional 5300 or so genes. Attempts to express multiple *Plasmodium* open-reading frames have been made in the past [18], but maintaining *Plasmodium* codon usage leads to lower levels of expression and low levels of intact product in commonly used hosts. It would be of major benefit to produce the entire proteome of the parasite re-coded for expression in bacterial and eukaryotic hosts in a system, such as the multi-site Gateway Technology (Invitrogen Life Technologies;

<http://www.invitrogen.com>), to allow cloning into many different vectors. With the new Gateway Open Architecture licensing policy, it is now feasible that these could be made available through mechanisms such as the malaria repository MR4 (<http://www.mr4.org>) and would allow laboratories worldwide to use their specific expertise on malaria genes of interest. It would also help laboratories in the developing world, hampered by limited resources and often excluded from the large collaborative networks, to take a more active role in the study of a parasite of particular interest to them. Using currently available technologies, this is an expensive exercise and would require many millions of dollars. Some form of prioritization could be used to select a subset of genes while we await improved oligonucleotide synthesis methods. Producing the entire parasite metabolome would be a worthwhile start, followed perhaps by the merozoite or pRBC 'surfaceomes'. These reagents would also be of use to larger groups such as the Structural Genomics of Parasitic Protozoa Consortium (<http://depts.washington.edu/sbpp>), which has had apparent difficulty in applying high-throughput methods to *Plasmodium*. At the time of writing this article, they had only been able to solve five crystal structures out of > 5300 proteins that comprise the *P. falciparum* proteome.

Dealing with data

As this process of elucidating protein function, the work of decades, continues, we will amass a vast amount of data about the parasite, including expression profiles derived under various conditions, proteome and interaction maps, and functional assignments. To this we can add data derived from independent studies of the human host and mosquito vectors. Integrating this to obtain new insights and therapeutic opportunities will be a massive informatics challenge. PlasmoDB [19], or some successor database, might be able to house these data and present them in a useful manner, but better methods of integrating the data are required. This is a challenge that faces all disciplines empowered by a genome sequence, hence this problem will probably first be solved outside the field of malaria and then applied to our own discipline. New methods of data integration and mining do not obviate the importance of primary data quality and we would stress the need for continuing curation of malaria genomic information, a pressing responsibility of the malaria community as a whole.

We lack methods for comprehending the effect on an organism of changing expression levels of several hundred genes simultaneously. The understanding of even more complex systems such as the host–parasite interaction will require a more quantitative approach. Modelling studies have often simplified the biology to make the mathematics easier [20,21], but they run the risk of making the study too artificial. Moreover, we should not allow the plethora of molecular detail to dominate our view of the malaria parasite itself. Our understanding of the fine morphological detail of the parasite also requires bolstering, and the application of modern imaging methods can provide much insight into its three-dimensional architecture. The recent work of Lawrence

Bannister *et al.* (Kings College, London, UK) to establish a comprehensive morphological atlas of the malaria parasite on which to place this multitude of new proteins, the 'ome for the proteome', is important for our continued understanding of the parasite as an infectious agent. Following a long period of concentration on training scientists in molecular disciplines, we are now experiencing a deficit of individuals trained in the area of cell biology and related techniques.

Systems biology

Much is made at present of the concept of systems biology [22]. Although widely perceived as a modern, revolutionary discipline, it is hardly novel and has been an established theme in biological research since the 1940s [23]. Systems biology comprises a multidisciplinary approach to solving biological problems by combining techniques that span the disciplines of the conventional sciences of biology, chemistry, physics, mathematics, computing and engineering, and those more recently established disciplines of molecular biology, genomics, bioinformatics and nanotechnology. Whether or not it is possible to systematize the performance of cross-disciplinary studies in malaria in this way, it is likely that a cross-disciplinary study of this sort could yield major insights, particularly by the use of more-sensitive analytical techniques in the human subject. Furthermore, a systems biology approach offers the chance of exposing experts in other disciplines to the fascinations and challenges of malaria, and potentially luring new talent into the field.

In vivo models

Although the rodent malaria model systems have served us well, one of the impediments to progress in malaria has been the lack of a robust and faithful model of all aspects of the human disease in readily accessible experimental animals. There are essentially two ways forward. First, to use our comprehensive knowledge of the human immune system and hemopoietic stem cell biology, together with the availability of immunodeficient mice, to consider reconstructing these animals with a fully human immune system. One could imagine that such animals could provide much better predictors of the immunogenicity of vaccine formulations, and it might even be possible to graft these animals with human hepatocytes and infect them with malaria parasites of humans. Potential drawbacks include the small size, increased fragility and shorter life-span of the mouse RBC, which might prevent the human malaria parasites developing properly, in addition to likely alterations in the interactions between fibroblasts, endothelial cells and immune cells in a mouse-human hybrid. Nevertheless, there is a certain seductive charm in the concept and it is certainly one worth exploring for its applications in many areas of infectious disease and autoimmunity research. The second way forward, and the one that we would favor in the shorter term, is to develop methods that can track parasite load, parasite secondary metabolism and host cell modifications, immune and hormonal responses in the human host in endemic areas. Often, only small sample volumes

are available, but advances in methodology and instrumentation will probably allow these to be analyzed in more detail. We can already see the beginnings of this in new methods for unbiased whole-genome amplification [24], although there will unquestionably be problems applying this to the high AT-rich genome of *P. falciparum* that has in the past proved difficult for standard PCR amplification protocols. It will be interesting to see whether the incorporation of these data into quantitative models will provide new ways forward in our understanding of mechanisms of pathogenesis, and the nature and modulation of host immunity in response to infection.

A coordinated approach

It is also pertinent to consider briefly collaboration versus competition within the malaria research community. Research is increasingly becoming the domain of 'centers of excellence' and large, well-funded collaborative groups. While the benefits and synergies of large, multidisciplinary teams compared with small groups or individual investigators cannot be denied, the question of what future there is for the individual investigator must be asked. The large programs do not publicize their experimental plans, making it difficult for small groups to know whether they might be in direct competition. Many small laboratories and scientists in disease-endemic countries with relatively poorly funded programs conduct malaria research. What involvement can these groups have in a post-genomic research landscape? Can they take part in a coordinated approach to the systematic evaluation of genes and how could this be organized? The establishment of better communication, possibly in the form of organized discipline-specific networks, represents one way to address this issue. We also strongly support an increased commitment to skills training of endemic-country scientists and would like to see an expansion of the workshop programs funded by the World Health Organization and United Nations Development program, National Institutes of Health, MR4 and the Wellcome Trust, which has focused on training courses in malaria methods, as well as new technologies such as microarrays, parasite transfection and bioinformatics.

Looking to the future

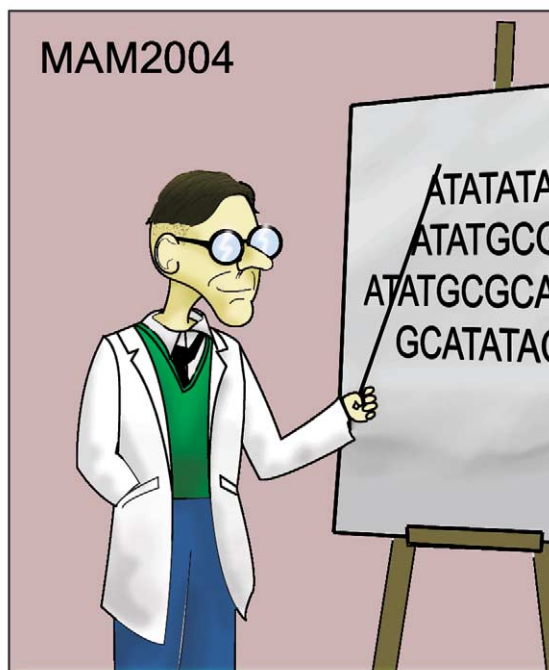
So, what might we expect to see in a Special Issue of *Trends in Parasitology* after the next Molecular Approaches to Malaria Meeting in 2008? The skeptics might say that what is most likely to be reported are yet more claims of cutting-edge research on the brink of a major breakthrough, and calls for more and greater funding. Ultimately, it is up to us to prove the skeptics wrong. Malaria research remains a boutique occupation, and the deployment of large-scale academic and industrial resources that occurred in the study of HIV infection and led to the development of more effective drugs has not yet happened in our field. Nevertheless, we as a research community must continue to believe that if we can devise effective measures, they will be deployed. Let us hope that the next four years brings us not only scientific progress, but also practical outcomes.

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References

- Noguer, A. *et al.* (1978) The malaria situation in 1976. *WHO Chron.* 32, 9–17
- Webster, D. and Hill, A.V. (2003) Progress with new malaria vaccines. *Bull. World Health Organ.* 81, 902–909
- Breman, J.G. (2001) The ears of the hippopotamus: manifestations, determinants, and estimates of the malaria burden. *Am. J. Trop. Med. Hyg.* 64, 1–11
- Gardner, M.J. *et al.* (2002) Genome sequence of the human malaria parasite *Plasmodium falciparum*. *Nature* 419, 498–511
- Holt, R.A. *et al.* (2002) The genome sequence of the malaria mosquito *Anopheles gambiae*. *Science* 298, 129–149
- Carlton, J.M. *et al.* (2002) Genome sequence and comparative analysis of the model rodent malaria parasite *Plasmodium yoelii yoelii*. *Nature* 419, 512–519
- Carlton, J. (2003) The *Plasmodium vivax* genome sequencing project. *Trends Parasitol.* 19, 227–231
- Bozdech, Z. *et al.* (2003) The transcriptome of the intraerythrocytic developmental cycle of *Plasmodium falciparum*. *PLoS Biol* 1, E5
- Kaiser, K. *et al.* (2004) Differential transcriptome profiling identifies *Plasmodium* genes encoding pre-erythrocytic stage-specific proteins. *Mol. Microbiol.* 51, 1221–1232
- Kappe, S.H. *et al.* (2001) Exploring the transcriptome of the malaria sporozoite stage. *Proc. Natl. Acad. Sci. U. S. A.* 98, 9895–9900
- Gardiner, D.L. *et al.* (2003) Malaria transfection and transfection vectors. *Trends Parasitol.* 19, 381–383
- Crabb, B.S. *et al.* (2004) Transfection of the human malaria parasite *Plasmodium falciparum*. *Methods Mol. Biol.* 270, 263–276
- Ho, Y. *et al.* (2002) Systematic identification of protein complexes in *Saccharomyces cerevisiae* by mass spectrometry. *Nature* 415, 180–183
- Meissner, M. *et al.* (2003) Establishment of a tetracycline-based inducible system for the control of gene expression in *Plasmodium falciparum*. *Exp. Parasitol.* 105, 58
- Cooke, B.M. *et al.* (2001) The malaria-infected red blood cell: structural and functional changes. *Adv. Parasitol.* 50, 1–86
- Cooke, B.M. *et al.* (2004) Malaria and the red blood cell membrane. *Semin. Hematol.* 41, 173–188
- Van Vliet, K.J. *et al.* (2003) The biomechanics toolbox: experimental approaches for living cells and biomolecules. *Acta Materialia* 51, 5881–5905
- Haddad, D. *et al.* (2004) Novel antigen identification method for discovery of protective malaria antigens by rapid testing of DNA vaccines encoding exons from the parasite genome. *Infect. Immun.* 72, 1594–1602
- Bahl, A. *et al.* (2003) PlasmoDB: the *Plasmodium* genome resource. A database integrating experimental and computational data. *Nucleic Acids Res.* 31, 212–215
- Gandon, S. *et al.* (2001) Imperfect vaccines and the evolution of pathogen virulence. *Nature* 414, 751–756
- McQueen, P.G. and McKenzie, F.E. (2004) Age-structured red blood cell susceptibility and the dynamics of malaria infections. *Proc. Natl. Acad. Sci. U. S. A.* 101, 9161–9166
- Kitano, H. (2002) Systems biology: a brief overview. *Science* 295, 1662–1664
- Wiener, N. (1948) *Cybernetics or control and communication in the animal and the machine*, MIT Press
- Hawkins, T.L. *et al.* (2002) Whole genome amplification—applications and advances. *Curr. Opin. Biotechnol.* 13, 65–67



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