local malaria cases in Costa Rica [1]. From 2016 onwards, the 7-day treatment was included in Costa Rica's national malaria norm [7]. This norm mandated the use of the 7-day treatment, already used by the Ministry of Health during malaria outbreaks, to all health centers administered by the Costa Rican Social Security Trust (Caja Costarricense de Seguro Social), the public trust in charge of administering universal health care in the country [7].

Lessons for Malaria Elimination in Mesoamerica and México

The experience with 7-day treatments was fundamental to accelerate the malaria pre-elimination stage in Costa Rica. In Mesoamerica, a region highly vulnerable to malaria and other vector-borne and parasitic diseases [14], only El Salvador, Belize, and Costa Rica are on track to achieve malaria elimination by 2020 [1]. Costa Rica was able to abruptly reduce the high malaria case burden of 2005, being the first nation to achieve the landmark of no local malaria cases for a full year in Mesoamerica in 2013, and without either passively or actively detected cases for 33 months until 2015. Thus, the Costa Rican experience is highly informative for other nations in Mesoamerica and México, where the regional malaria burden only accounts for less than 1% of the global burden, as most cases are due to chloroquine-sensitive *P. vivax* parasites [8,14]. Costa Rica's experience illustrates how focalized MDAs, combined with a malaria treatment shift that minimizes the likelihood of *P. vivax* relapses, could become a major tactic in the strategy for malaria elimination. Data from Costa Rica show that 7-day treatments are a good choice for MDAs, given their relatively short duration, which increases full adherence [7]. The supervised implementation of 7-day treatments by trained technical staff, as done by NPIVM inspectors in Costa Rica, can help to monitor adverse reactions to primaquine in populations with a low frequency, but imprecise estimates, of G6PD mutations associated with primaquine-induced hemolytic anemia. Finally, Costa Rica's tactics can help to accelerate malaria elimination in parts of the world with key epidemiological similarities: a low frequency of primaquine-sensitive G6PD mutations and chloroquine-sensitive malaria parasites.

Resources

[i]www.paho.org/hq/index.php?option=com_content&view=article&id=2632:2010-interactive-malaria-statistics&Itemid=2130&lang=en

[ii]www.ministeriodesalud.go.cr/index.php/biblioteca-de-archivos/centro-de-informacion/material-publicado/boletines-1/boletines-vigilancia-de-la-salud

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Spotlight

Tying up Loose Ends in the Malaria Antigenic Variation Story

David E. Arnot [1,*]

A recent paper (Zhang *et al.*, *PLoS Biol.*, 2019) shines remarkable new light onto the malaria antigenic variation story. Using CRISPR/Cas9-targeted chromosome breaks and long-read whole-genome sequencing, they followed the fate of detached subtelomeric *Pf*EMP1/*var* genes and demonstrated that these initiate cascades of recombination at sites far from the original break.

The *Pf*EMP1 variable surface antigens of *Plasmodium falciparum* are erythrocyte membrane adhesion receptors encoded by 50–90 very diverse but related *var* genes [1]. The majority of *var* genes are in subtelomeric regions of the 14 chromosomes of *P. falciparum*. Pathology caused by falciparum malaria is directly related to shifts in *var* expression because different *Pf*EMP1s bind different host receptors and organs, leading to varying consequences for the host. Antigenic switching aids survival in the face of cytoadhesion-blocking antibodies, establishes chronic malaria, and maintains the infectious reservoir. Yet *P. falciparum* is just a protozoan that does not even know it is a parasite, let alone that it must evade antibodies blocking its escape routes from the blood

circulation onto endothelial surface receptors.

Antigenic variation research is thus continually facing the question of how an unintelligent genetic program plays such a chess-like game with the human immune system. The framework of the coevolved system is clear enough – immunity-evading diversity is multiplied using recombination, and recombination must maintain functional adhesion receptors. But what fundamentally drives *P. falciparum* antigenic variation? What induces it, and what happens during and after expression switches?

Recombination is fundamental to structuring the system. *Pf*EMP1 proteins may seem near-infinitely diverse but each is a recombined composite of not many more than 628 polypeptide 'basic homology blocks' of 25–200 amino acids [2]. A strong relationship has been found between homology block boundaries, base-paired internal DNA secondary structures (DSSs, Box 1), and 'hot spots' for *var* recombination [3]. Clone family trees scanned by whole-genome sequencing also demonstrated that composite *var* genes largely derive from homologous recombination (HR, Box 1) between *vars* on different chromosomes during asexual, mitotic replication of the parasite [4,5], although meiotic recombination between different parental clones also occurs [3].

A new paper by Zhang *et al.* [6] reveals something remarkable when repair of targeted double-strand breaks (DSBs, Box 1) generated by CRISPR/Cas9 is followed in detail. Zhang *et al.* engineered a DSB into a subtelomeric region of *P. falciparum* chromosome 12 containing three *var* genes. Guide RNAs positioned the DSB upstream of two *vars* and one linked *rifin* gene. Initial transfections used the Cas9 expression construct, cotransfected with an episomal plasmid containing a 'repair template' of sequences identical to the sequences flanking the targeted break sites plus a dihydrofolate reductase (*dhfr*) gene for drug selection as well as the guide RNA expression cassette.

As previously observed, when provided with a homologous template for DSB repair, transfected *P. falciparum* uses HR to repair the break, preserving reading frames and chromosomal structure with minimal disruption and no detectable rearrangements elsewhere in the genome [7]. But when Zhang *et al.* did not provide a template for HR in the transfections, CRISPR/Cas-induced breakpoints were 'healed' by *de novo* addition of telomere repeats. This stabilizes the broken chromosome end. However, although 'lost ends' can be reconstituted by telomeric gene conversions, this will lead to telomere homogenization, and subtelomeric *var* diversity will be lost. The more attractive hypothesis thus becomes that detached DNA fragments generated by subtelomeric DSBs, which cannot be repaired by HR, become not 'lost' but 'free' – free to recombine with other reasonably homologous *var* genes at distant ectopic sites. Preliminary PCR amplifications indicated that the detached sequences indeed still existed somewhere in the transfected parasite genome. The new data of Zhang *et al.* reveal the fate of the broken chromosome end and its missing *var* genes.

Their search for the lost *vars* utilized advanced sequencing technology which now achieves single-molecule, long reads of more than 10 kilobases. High-resolution genome reconstruction thus becomes much simpler, and Zhang *et al.* could accurately scan three lines, each with different chromosome 12 truncations. As would be expected, some recombination proximal to the chromosome 12 DSB occurred. But chromosome 12 DSBs also appeared to have somehow triggered recombination between bystander *var* genes on chromosomes 6 and 13, in each independently derived line. How can this be?

Assuming all chimeric sequences to be the results of HR, Zhang *et al.* demonstrated a very plausible recombination scenario. The released DNA fragment from chromosome 12 initiated the observed recombination between a now extrachromosomal *var* and a chromosome 6 *var*. This created a new chimeric *var* and transposed the end of chromosome 12 onto the end of chromosome 6, stabilizing the loose end of chromosome 12, but detaching the

Box 1. Some Features of Recombination in *Plasmodium falciparum*

Homologous Recombination (HR)

A form of recombination requiring donor sequences to have some sequence similarity as well as physical proximity. *P. falciparum* is unusually dependent on HR as the 'classical' nonhomologous end-joining (NHEJ) recombination pathway enzymes have been lost from its genome. It seems likely that this is adaptive and its dependence on HR repair accounts for the paucity of 'pseudo-genes' in the *P. falciparum* genome.

Double-Strand Breaks (DSBs)

DNA DSBs can occur during replication, mitosis, or meiosis, or be induced, for example, by X-rays or mutagens. The beauty of the CRISPR/Cas9 system is that it allows experimenters to choose where to place the DSB, using sequence homologies to position 'guide RNAs' for the Cas 9 endonuclease.

DNA Secondary Structures (DSSs)

If the DNA double helix 'melts' to break apart hydrogen-bonded base pairs, it is possible to form intrastrand 'hairpin' or 'cruciform' secondary structures. The A+T-rich base composition of *P. falciparum* (82% A+T overall), which can reach ≫90% A+T in noncoding DNA, facilitates helix melting. The enhanced DSB-promoting activity and recombinogenic potential of DSSs formed by quasi-palindromic DNA sequences is well established, although detailed mechanisms remain unclear.

chromosome 6 end with its *var* gene and two *rifin* genes. This event was, in turn, stabilized by the chromosome 6 *var* recombining with a chromosome 13 *var*. The apparently unrelated chromosome 6/13 *var* recombination is thus seen to be a consequence of a recombinational cascade initiated by a DSB in chromosome 12.

The implication is that the structure and functioning of the antigenic variation system of *P. falciparum* have evolved from activated DNA repair pathways. As Zhang *et al.* note, any chromosome break generating free DNA fragments is recombinogenic, and cascade-type processes are always possible. DSB-initiated adaptive recombinational cascades have been observed in cancer cells [8] and in that model for antigenic variation, trypanosomes [9]. Another recent paper makes the interesting and somewhat parallel case that antimalaria drug-induced metabolic stress increases the rate of *P. falciparum* DSBs (here associated with intragenic DSSs) causing genome duplications to facilitate drug resistance via target protein overexpression [10].

The emerging theme, greatly clarified by the article from Zhang *et al.*, is that the *P. falciparum* genome, intragenic as much as genic, has evolved to respond to stress by exploiting evolutionarily ancient DNA-repair pathways. The positioning of a DSB, which mainly occurs during mitotic DNA replications in host erythrocytes, is not random but DSS-determined. Recombination hotspots, constraining outcomes, have evolved. Adaptive genomic plasticity underlies the capacity of *P. falciparum* to sometimes stay one step ahead of a very dangerous host capable of simultaneously cooking it with fevers, devouring it with phagocytes, smothering it with antibodies, and poisoning it with drugs. During 'stressful' DNA replication rounds, increasing numbers of DSBs at nonrandom positions initiate multiple mitotic recombinational cascades among a vast intraerythrocytic parasite population to generate recombinant progeny which might succeed in attaining a new level of fitness, and survive.

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Spotlight

Post-Kala-Azar Dermal Leishmaniasis as a Reservoir for Visceral Leishmaniasis Transmission

Epke A. Le Rutte,[1,2,3,*] Eduard E. Zijlstra,[4] and Sake J. de Vlas[1]

Post-kala-azar dermal leishmaniasis (PKDL) is a parasitic skin infection which can occur after visceral leishmaniasis (VL). Recent xenodiagnosis studies (Mondal *et al.*, *Clin. Infect. Dis.*, 2018) have uncovered the infectiousness of PKDL. When including this in a transmission model, PKDL cases appear as an important reservoir of infection, likely frustrating the VL elimination efforts on the Indian subcontinent.

Kala-azar, commonly known as visceral leishmaniasis (VL), is a neglected tropical disease that has been targeted on the Indian subcontinent (ISC) for elimination as a public health problem by 2020, that is, a reduction of incidence to <1 VL case per 10 000 of the population per year at (sub)district level. *Leishmania donovani* protozoa are transmitted by female sand flies and infect humans, who mostly remain asymptomatic. However, a small percentage (1–10%) develop symptomatic VL, which is fatal if left untreated [1]. After recovering from VL, about 2.5–20% of patients develop post-kala-azar dermal leishmaniasis (PKDL), a nonlethal skin condition that can last for years and appears in two main forms: nodular or macular [2]. The interventions to achieve the 2020 elimination target focus on timely diagnosis and treatment of VL cases, and vector control through indoor residual spraying of insecticide.

A crucial knowledge gap in the transmission dynamics of VL is the reservoir of infection. It is largely unknown if, and to what extent, asymptomatic and PKDL cases contribute to transmission, as has long been highlighted by the PKDL Consortium as one of the key topics for future research [3]. Infectious PKDL cases could be a long-lasting source of transmission and thereby frustrate elimination efforts. We have recently explored the significance of these unknowns using two mathematical