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Pathogen evolution issues in genetically modified mosquito vector strategies

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Abstract

In this paper, pathogen evolution will not be considered in the extended concept of adaptation through mutability, heredity and long-term adaptation through speciation. In contrast, the main issues deal with aspects related to polymorphism and population diversity that arise by selection processes and how these may influence vector–pathogen–human relationships. Three aspects related to these relationships will be presented in order to discuss the possibility that a reduction of the vector capability to transmit malaria could result in the selection of parasites. First, aspects of population size and diversity of *Plasmodium* (with emphasis on *P. falciparum*) relevant to epidemiology and host interactions will be presented. Next, *Plasmodium*–vector molecular interactions, determinant of infectivity, will be reviewed; these could determine the efficacy of a trait introduced by the genetic modification of the mosquito and/or could result in selection of parasites resistant to the trait. Finally, the possibility of virulence shifts in pathogens as a result of the genetically introduced traits in mosquitoes will be discussed.

Keywords: evolution; *Plasmodium*; population; mosquito vectors; molecular interactions; virulence shifts

Plasmodium population size and diversity

Genetic variation in malarial parasites has practical significance for control strategies based on the elimination of parasite transmission. Highly polymorphic molecular targets in the parasites could limit the efficacy of the mechanism introduced for their elimination. On the other hand, selective pressures imposed by the intervention could generate parasite mutants in highly plastic genome populations much easier than in reduced genetic diversity ones. There is an extensive contradictory literature on the variability of *Plasmodium falciparum*. The almost complete absence of silent nucleotide substitutions in coding sequences of nine genes (including the circumsporozoite protein (CSP)) and 25 introns of eight independent parasite isolates was interpreted to reflect a recent origin of the world's parasite populations (Rich, Hudson and Ayala 1997; Volkman et al. 2001); this would be inconsistent with extant polymorphisms in genes of ancient proteins (MSA-1, AMA-1) (Verra and Hughes 2000) as well as CSP, which originated before *P. falciparum*

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split from *P. reichenowi* (Hughes 1992; Dubbeld, Kocken and Thomas 1998; Okenu, Thomas and Conway 2000).

Others have identified over 400 polymorphic sites including 238 single-nucleotide polymorphisms (SNPs) and 165 microsatellites (Mu et al. 2002), and an effective population size of (N_e) 10^5 for the past 300,000-400,000 years was calculated through the analysis of the DNA sequence of 23 nuclear protein-coding loci on the basis that these were not subject to selection (Hughes and Verra 2001). This estimation is consistent with that obtained using microsatellite data ($N_e = 10^2$ - 10^4) (Anderson et al. 2000), indicating a large population with extensive possibilities for variation.

Several possible explanations point towards unique characteristics of the *Plasmodium* biology and gene protein structure to conciliate an extensive polymorphism in genes with specific functions and low rates on SNPs in non-coding regions. Compared to homologues in other organisms, *P. falciparum* proteins are longer because they contain low-complexity regions (Pizzi and Frontali 2001), these regions usually form tandem repeats located at non-globular domains of the open reading frames. *P. falciparum* genes have high A-T contents, a feature more pronounced in the repeat regions imposing limitations to the structure of transfer RNA, thus limiting the amount of nucleotide substitution that could maintain the viability of the organism (Forsdyke 2002).

On the other hand, analysis of polymorphism in non-repeat regions in the CSP protein indicated that these are not randomly distributed, but restricted to B- and T-cell epitopes (Rich, Hudson and Ayala 1997) indicating that these result from strong natural selection. Interestingly, haplotypes in the two non-repeat regions of the protein correlate with one another, but not with the intervening repeat region, which may indicate the clonality of the parasite population structure.

Beside an extensive effective population size, the plasticity of the *Plasmodium* genome and the effect of selection were documented by analysing 342 highly polymorphic microsatellite markers (Wootton et al. 2002) in chloroquine-resistant parasites. This analysis indicated at least four geographically different founding events, and extensive linkage disequilibrium surrounding the *ofcrt* (resistance-encoding gene) that occurred over only 20-89 parasite sexual generations (~6-30 years).

Thus, for the purpose of this discussion we can state that the *P. falciparum* parasite origin is ancient with a large effective population size. Although the parasite has stringent constraints with respect to mutation, its plasticity confers this pathogen the possibility to adapt to a variety of conditions in its vertebrate and insect hosts. This plasticity is reflected in the range of mosquito genera that could transmit *Plasmodium* and the wide range of anophelines that do transmit malaria to humans (Bruce-Chwatt 1985); moreover, it indicates that during evolution accumulated mutations in the parasites have enabled them to explore and exploit new niches with different conditions requiring molecular modifications for the interactions with the complex histological structure and defence responses of mosquitoes.

***Plasmodium*-vector molecular interactions as determinants of infectivity**

Much information on antigenic polymorphism and antigenic variation exists for parasite stages present in the vertebrate host, but little is known about possible adaptations to modulate the parasite interactions with their mosquito vectors. Understanding the biology of ookinete and sporozoite invasion and survival is

necessary to design the topological and temporal expression of introduced resistance genes. However this will not be examined at length here, yet some simplifications will be necessary to present some aspects of parasite development for the sake of a concise discussion.

Malaria parasites develop in a complex, compartmentalized milieu in the mosquito (Shahabuddin and Costero 2001). Survival in the blood meal bolus imposes a bottleneck in the ingested population. Invasion of the midgut epithelium and salivary glands requires molecules specialized for motility, epithelium recognition and survival. Thus the main characteristics of molecules required for the interaction of parasites with their host relevant for our discussion are: a) the variability of those molecules involved in cell recognition for invasion, and b) their possible variations to avoid the immune response.

Around 10-15 parasite molecules have been identified on the surface of ookinetes, but two proteins, P25 and P28, predominate and are very similar in rodent, bird and human malaria parasites (Kumar and Carter 1985; Kaslow et al. 1989; Paton et al. 1993; Tsuboi et al. 1998). These proteins, containing epidermal growth-factor domains, protect ookinetes within the midgut contents (Grotendorst and Carter 1987), and participate in interactions with the peritrophic matrix (Sieber et al. 1991) and the basal lamina (Vlachou et al. 2001). The most interesting characteristic of these proteins relevant for our discussion is that they have partially redundant functions and the exclusion of only one of them does not abrogate invasion (Tomas et al. 2001). Molecule receptors on the midgut surface could be sialic-acid-like carbohydrates (Zieler, Nawrocki and Shahabuddin 1999), but other glycoproteins with N-acetylglucosamine residues have been implicated (Ramasamy et al. 1997). Taken together these indicate that, as in the case of merozoites, parasite mosquito stages have developed mechanisms to better exploit variable conditions in potential mosquito vectors.

Non-synonymous nucleotide substitutions were documented in two proteins participating in the interaction of sporozoites with mosquito salivary glands and hepatocytes: thrombospondin-related anonymous protein (TRAP) and CSP (Hughes 1991; Hughes and Hughes 1995) might be the result of immune pressure in the vertebrate host. However, regions of genetic polymorphism have also been identified in both P25 and P28 molecules (Tsuboi et al. 1998), indicating that although these proteins are not under the immune pressure of the vertebrate host, genetic variation occurs; whether or not this has an effect on protein function and parasite selection resulting from unidentified mosquito factors remains unknown.

Except for sporozoites that remain for relatively prolonged periods in salivary-gland ducts, developing *Plasmodium* in mosquitoes stay for a short while inside the individual mosquito compartments. These parasites are not under the immune pressure faced by blood stages (Bull 1994). These stages have developed antigenic variation mechanisms (Beeson and Brown 2002; Recker et al. 2004; Craig and Scherf 2001; Blythe, Suretheran and Preiser 2004). Nevertheless, parasites encounter diverse protective mechanisms in mosquitoes, some of which render most anopheline species resistant to malaria infection (Bruce-Chwatt 1985); among those susceptible species, very few mosquitoes are found infected in the field (Haji et al. 1996). Even more, those that are reputedly good vectors block more than 99.9% of ingested parasites (Vaughan, Noden and Beier 1992).

As mosquito defence mechanisms are multiple, and their induction and regulation have only recently begun to be deciphered, these mechanisms will only be mentioned here to highlight the possibilities of parasite evasion mechanisms. Nitric-oxide

induction during ookinete migration through the epithelium induces destruction of the invaded cell (Han et al. 2000) and probably reduces infection, but surviving parasites may have evolved detoxification mechanisms, as it occurs with superoxide dismutase, that block oxygen species (Bécuwe et al. 1993). Interestingly, two C-type lectins induced in mosquitoes during parasite invasion prevent ookinetes from developing into oocysts by inhibiting parasite melanization (Osta, Christophides and Kafatos 2004). Concomitantly, immune responses (Richman et al. 1997; Dimopoulos et al. 1998; Gorman, Andreeva and Paskewitz 2000; Vizioli et al. 2001) are induced during malaria parasite development in mosquitoes, the *LRIM1* gene is activated during ookinete invasion and this is probably responsible for the activation of the innate immune response via TOLL-like receptors (Osta, Christophides and Kafatos 2004). Theoretically, surviving parasites may avert these defence mechanisms by lacking surface protein determinants that stimulate immune responses, and are therefore not recognized by the immune system, or secrete molecules that interfere with the immune response (Beerntsen, James and Christensen 2000).

Should we worry about mutation in the pathogens that could enable them to escape the control mechanisms introduced in the mosquito vector? There are very few empirical data that address the question of “what parasite traits are being selected in the process of natural selection?” or “which phenotypes or genes are targets of selection?”. A few theoretical models address the mechanisms (including mutation or survival strategy, recombination or reproductive strategy) by which variations are generated and persist in *Plasmodium* populations. It is evident that the possibility of avoiding the newly introduced resistant traits in mosquito vectors will depend on the nature of these traits. The capabilities of parasites to avert the new weapons should be inferred by the extensive variability in mosquito susceptibility to malaria parasites, indicative of the wide possibility of strategies these ancient parasites possess.

Virulence shifts in pathogens as a result of the GM mosquitoes

Virulence may be defined as the severity of the effect of infection on host mortality. In the case of *Plasmodium*–vector interactions, the pathogen virulence will have an effect on lifetime reproductive success (survival and fecundity) of the invaded mosquitoes. The general theory on the evolution of virulence assumes that selection tends to increase the parasites’ basic reproductive rate (R_0). If the rate of transmission is linked to virulence, selection may result in intermediate levels of virulence, but ever-increasing virulence may also result (Anderson and May 1979). Individual selection within parasite populations will favour whatever level of virulence maximizes their R_0 (May and Anderson 1983a). When competitive exclusion occurs among populations, only the strain with maximum R_0 can survive under general conditions (Bremermann et al. 1989), and selection will always favour the most virulent strain (Bremermann and Pickering 1983). On the other hand, factors determining the effect of the infection on the host, also determine the final outcome on the evolution of virulence.

Conventional wisdom (May and Anderson 1983b), supported by empirical observations (Levin and Eden 1990), states that virulence evolves towards less host-harmful parasites. But trade-off between transmission and parasite-induced host mortality leads towards an intermediate level of virulence (the enlighten theory). This is also predicted by simple models (Lenski and May 1994), and stable virulence will occur when the increased transmission becomes increasingly costly in terms of increased virulence. These theoretical considerations should be taken into account in

assessing the possibilities of shifts in virulence in natural infections and in GM vectors.

Plasmodium infections in mosquitoes are present as a mixture of parasite genotypes (Taylor, Walliker and Read 1997). When clones share resources the population dynamics of individual clones are affected by the presence of the others (Read and Taylor 2001). Competition can affect transmission success (fitness) of individual clones, thus shaping the evolution of virulence. This competition will also determine the fate of new mutants arising in the course of infection. Very little is known about parasite clonal competition in mosquitoes, but some insights could derive from observations in infections in humans. The number of clones present in older children and adults has no effect on parasite titres (Smith et al. 1999) indicating that clonal densities within hosts are not regulated independently, but density-dependent clonal diversity also occurs (Arnot 1998). Also, fewer clones are present in symptomatic patients than in asymptomatic infections, indicating competitive suppression (Mercereau-Puijalon 1996). Finally low parasite turnover in areas of low malaria transmission indicates that competitive exclusion also occurs (Daubersies et al. 1996). Thus, it is likely that clonal competition will also occur among parasites developing in mosquitoes (Taylor, Walliker and Read 1997).

In infections with related parasite strains, evolutionary stable virulence occurs if there is a trade-off between virulence and infectivity (Frank 1992). The final outcome of altering the parasite's genotype composition as a result of diminishing the force of infection (of some clones) of parasites in genetically modified mosquitoes will depend on whether virulent parasite species/strains will adaptively adjust their fitness in response to the change in mosquito genetic makeup, in the same way as they do in natural vector populations, and whether the selected parasites clones and the underlying clonal competition will increase or decrease transmission. More virulent strains may have intra-host competitive advantages, but may kill the host, while less virulent strains may have an inter-host advantage, because they are less harmful to the host and are transmitted for longer periods (Nowak and May 1994). We know little about fitness differences among *Plasmodium* populations across different endemic zones or even in the host. The same occurs on the ability of clones to produce gametocytes (that reflects parasite fitness) and to infect humans.

In nature, the hosts typically live in partially structured populations with transmission events occurring locally. The epidemiology of malaria could be considered a complex case of metapopulation ecology (Anderson 1991). Ecological patches are represented by areas of disease transmission, and each vertebrate and mosquito hosts as subsections of these patches. This puts a limit on the transmission rates of pathogens even without a trade-off with virulence (Rand, Wilson and McGlade 1994; Haraguchi and Sasaki 2000). A further clustering of resistant hosts (introduced by transgenesis) may have important implications for the invasion of recently evolved pathogen strains and their relative fitness. A pathogen that is transmitted very quickly may be more aggressive not only to non-resistant individuals but probably also to those with the resistant trait (Boots, Hudson and Sasaki 2004).

On the other hand, inhibition of transmission by the introduction of GM resistance traits in mosquitoes results in destruction of some but not all the ecological patches. Ecological models of competition indicate that patch destruction could lead to an increase in the number of patches, occupied by inferior parasite competitor (reviewed by Read and Taylor 2001). The outcome could be an increase in disease prevalence. But all this will depend on the established clonal competitiveness resulting from the intervention.

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