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An Analysis on Molecular Evolution and Systematics of Rodent Malaria Parasites

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Abstract – Over the last 6 decades, rodent Plasmodium species have become key model systems for understanding the basic biology of malaria parasites. Cell and molecular parasitology have made much progress in identifying genes underpinning interactions between malaria parasites, hosts, and vectors. However, little attention has been paid to the evolutionary genetics of parasites, which provides context for identifying potential therapeutic targets and for understanding the selective forces shaping parasites in natural populations. Additionally, understanding the relationships between species, subspecies, and strains, is necessary to maximize the utility of rodent malaria parasites as medically important infectious disease models, and for investigating the evolution of host-parasite interactions.

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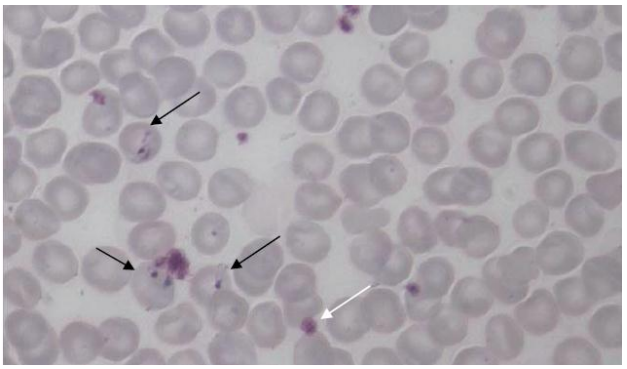
INTRODUCTION

After ingestion of infectious *Plasmodium* gametocytes by the mosquito, motile ookinetes develop in the midgut lumen and traverse the chitin-containing peritrophic matrix (PM), the microvillus-associated network, and the midgut epithelium to form sporozoite-producing oocysts on the hemocoel side of the midgut. After the demonstration that ookinetes secrete multiple chitinase activities, two distinct *Plasmodium* chitinase genes were isolated. The first was isolated from the human malaria parasite *Plasmodium falciparum* (*PfCHT1*), while the second was found in the avian malaria parasite *Plasmodium gallinaceum* (*PgCHT1*) . The primary structures of these two chitinase genes are markedly different: *PgCHT1* encodes putative amino-terminal proenzyme and carboxy-terminal chitin-binding domains, which are both absent in *PfCHT1. P. gallinaceum* secretes a second chitinase activity provisionally named PgCHT2, believed to be orthologous to that encoded by *PfCHT1* based on its molecular mass and physiological properties (pH optimum and sensitivity to the chitinase inhibitor allosamidin), and it may have additional chitinase activities.

The *Streptomyces*-produced molecule allosamidin is a 622-dalton pseudo-oligosaccharide that inhibits *Plasmodium* chitinase activities in vitro (10, 14, 15). Moreover, the presence of allosamidin in an infected blood meal inhibited oocyst formation of *P. gallinaceum* in *Aedes aegypti* and of *P. falciparum* in *Anopheles freeborni*, a process that was reversed when the PM was prevented from forming by the addition of exogenous chitinase to the blood meal (10). Although these inhibitor studies identified *Plasmodium* chitinases as potential malaria transmission-blocking targets, dissection of the roles of the individual chitinase activities in mosquito infection remains a prerequisite for a rational chitinase-based transmission-blocking vaccine or drug design.

P. chabaudi was first isolated from the blood of a shiny thicket rat in the Central African Republic in Africa, by I. Landau and A. Chabaud in 1965. Two subspecies have been defined, *P. chabaudi chabaudi* and *P. chabaudi adami*. The parasite is readily grown in laboratory mice and rats, where it shows a preference for mature red blood cells are synchronous with a periodicity of 24 hours. The parasite may be transmitted in the laboratory by *Anopheles stephensi* mosquitoes.

The following photographs show *P. chabaudi* strain CB parasites in mouse blood. All photos are at x100 magnification, although some have been digitally enlarged.



P. chabaudi day 2 post-innoculation (pi). Infected red blood cells (RBCs) are marked with black arrows. These are early “ring-stage” trophozoites, and consist

of a dark purple nucleus and lighter, blue coloured cytoplasm, often forming a ring around the nucleus. The darker coloured objects (one is marked with a white arrow) are platelets.

The species of *Plasmodium* infecting rodents belong to the subgenus *Vinckeia*, which contains all the non-primate mammalian parasites. These parasites are described as follows (Garnham, 1964) : Vinckeia, *sub gen. n, includes species of malaria parasites found in various mammals below the simian level. The erythrocytic schizont does not fill the corpuscule and gives rise to eight or fewer merozoit.es, True stippling is absent. Gametocytes are spherical and sporogony proceeds in anopheline mosquitoes. Exo-erythrocytic schizogony is rapid, taking three days or less, and secondary tissue stages are rare or missing.*

MOLECULAR SYSTEMATICS

The malaria parasites are included within the order Haemosporidia, phylum Apicomplexa. At least 15 genera of haemosporidians have been placed into the family Plasmodiidae. The best-known genera are *Plasmodium*, which is the cause of human malaria (more on those species below), and *Haemoproteus*, found primarily in birds.

The common term, "malaria parasites," is controversial in its use. Malaria is a disease, not a parasite. Thus, it is not appropriate to write or speak of "malaria infecting a bird." So, the parasites should be referred to as "malaria parasites" just as we might refer to "the cold virus." But, malaria is a human disease, with characteristic symptoms. Infection causes pathology in other vertebrate hosts, but is it the same disease? Also, should the term be restricted to *Plasmodium*, and not to other genera in the family? We prefer that common names be based on monophyly, but the choice of a node on a phylogenetic tree to define any higher-level taxon (above species level) is subjective. We therefore call all genera in the family, the "malaria parasites," and include *Leucocytozoon* in this group. *Leucocytozoon*, though, could well be placed into a separate family because it is an outgroup to all the others studied to date.

The genera are defined by life history traits. Picking the two best known, *Plasmodium* and *Haemoproteus*, illustrates this point. *Plasmodium* when it first enters a vertebrate host, undergoes a bout or several bouts of asexual replication in solid tissues, then emerges in the blood to infect blood cells.

Asexual replication continues, and eventually non-replicating gametocytes are produced. The male and female gametocytes are the stages that are transmitted to the biting vector where sexual recombination occurs. *Haemoproteus* is similar except all the asexual replication in the vertebrate occurs in the solid tissues, and only gametocytes are seen in the blood. This must have been a major evolutionary step, and some authors suggest that *Haemoproteus* is the ancestral life cycle.

A more extreme difference is seen in *Hepatocystis*. Again, only gametocytes are seen in the blood, but they are huge, filling the entire red blood cell, and do not look at all like those of *Plasmodium* or *Haemoproteus*. The asexual stages are found in the liver (thus the generic name), and the dividing cell is huge, even visible to the naked eye.

How are these genera related? Molecular phylogenies should reveal their relationships. But, what gene should be used? A rapidly evolving gene would be useless because the relationships are deep, and the splits occurred very long ago. *Plasmodium* is found in lizards in North and South America, Australia, Asia, and Africa, and thus the genus must antedate the split of the continents. A rapidly evolving gene would suffer repeated mutations at all sites that are able to tolerate changes, thus becoming "saturated" over such long periods of time, and any phylogenetic signal would be lost.

The gene first used in early studies of the molecular phylogeny of malaria parasites was the small subunit of the ribosomal RNA gene. This is a gene widely used to recover deep nodes on phylogenetic trees. However, in *Plasmodium* and related parasites, there are multiple copies of this gene, which become active during different stages of the life cycle (asexual stage, sexual stage, etc.). These copies of the rRNA gene in the parasites do not evolve together, and thus diverge over evolutionary time. Susan Perkins of the American Museum of Natural History has been studying this gene in detail for the entire phylum and concludes that it is a poor key to the phylogeny of the parasites.

Sex ratios in the rodent malaria parasite In order for malaria parasites to transmit to new vertebrate hosts, a round of sexual reproduction must be undertaken in the mosquito vector. Sexual stages, termed gametocytes, are produced from the parasite's asexual cycle and are the functional equivalents of males and females. Within 20 min of being taken up in the bloodmeal of a mosquito, the gametocytes have differentiated into gametes (Micks, de Caires & Franco, 1948; Billker et al. 1997). Each female gametocyte produces 1 female gamete and each male gametocyte can produce up to 8 male gametes (Sinden, 1975; Sinden et al. 1978; Janse et al. 1989; Schall, 2000). Male gametes are motile (each has a flagellum), and can fertilize female gametes either from the same clonal lineage (genotype), or outcross with other genotypes. The fertilized zygotes undergo several stages of asexual replication before migrating to their vector's mouthparts, ready to infect a new host. The gametocyte sex ratio (defined as the proportion of male gametocytes) is an important factor in determining how well a parasite genotype maximizes its genetic representation in the population of new infections (Robert et al. 1996; Paul et al. 2000;

Schall, 2000; Paul, Brey & Robert, 2002). Recently there has been an increased interest in using evolutionary theory to explain the sex ratios observed in malaria parasites (reviewed by West, Reece & Read, 2001; Read et al. 2002). Theory predicts that the sex ratio (r*), should be related to the inbreeding rate by the equation r*=(1–F)/2, where F is Wrights coefficient of inbreeding (the probability that 2 homologous genes in 2 mating gametes are identical by descent;Dye & Godfray, 1993; West, Smith & Read, 2000; Nee, West & Read, 2002). Whilst this relationship has enabled a broad-scale understanding of the sex ratios observed in malaria and related Apicomplexan parasites, there are discrepancies that demand elucidation (West et al. 2001; Paul et al. 2002). To date, there has been little work on how appropriate standard empirical measurements of apicomplexan sex ratios are for sex allocation theory (Read et al. 2002). Here, we address this issue.

First, we tested the accuracy of standard methods for estimating gametocyte sex ratios. The sex of gametocytes is usually assigned by examination of thin blood smears stained with Giemsa's solution. However, this methodmay overestimate the proportion of female gametocytes if maturing male gametocytes resemble females and are sexed wrongly from thin blood smears (Schall, 1989; Dearsly, Sinden & Self, 1990). Sex ratio estimates made with specific molecular markers can be significantly less female biased than when made with thin blood smears although it should be noted that no concurrent comparison has been made (Ranford-Cartwright et al. 1993; Smith et al. 2000, but see Silvestrini, Alano & Williams, 2000). In addition, sex ratio estimates can be much more female biased than expected – in extreme cases there cannot be enough males to fertilize all of the female gametes (Burkot, Williams & Schneider, 1984; Pickering et al. 2000). We tested this possibility using the rodent malaria parasite Plasmodium chabaudi, by comparing the sex ratios observed in thin blood smears with sex ratios observed using a method in which gametocytes are partially allowed to differentiate into gametes to reveal their sex more clearly.

Secondly, we tested the assumption that the mortality rate of male and female gametocytes is equal in P. chabaudi. Sex ratio theory is concerned with predicting the sex ratio at the point when sexual differentiation occurs (defined as the primary sex ratio), and differential mortality results in the observed sex ratio (the secondary sex ratio), differing from the primary sex ratio. Primary and secondary sex ratios may differ for a number of non-exclusive reasons.

In most organisms, females live longer than males (Owens, 2002). For malaria parasites, sex-biased mortality could occur in a number of ways. Male and female sex-specific antigens exist (Severini et al. 1999; Eksi & Williamson, 2002), so that male and female gametocytes could be killed by host immunity at different rates (Paul et al. 2000, 2002; Reece & Read, 2000). In species that sequester there may be sex specific rates for sequestration in the capillaries.

If the mortality rate is greater for male gametocytes, natural selection could favour a less female biased sex ratio for a given level of inbreeding to insure there are enough male gametes in the bloodmeal to fertilize all the female gametes (Paul et al. 2000, 2002; West et al. 2001, 2002; Gardner, Reece & West, 2003).

METHODOLOGY

General experimental methods-

We maintained 40 female MF1 mice, aged 10–12 weeks (Harlan-Olac, UK), on a 12 h light/12 h dark cycle, ad libitum diets (41B, Harlan-Teklad, UK) and ad libitum water containing 0.05% para-aminobenzoic acid (PABA) (to enhance parasite growth). Twenty mice were assigned randomly to the P. chabaudi experiment and 20 were assigned to the P. vinckei experiment. Within each experiment, mice were randomly assigned to cages of five and within each cage randomly assigned to control and experimental (Zprobing) treatments. Both control and experimental mice were infected via intra-peritoneal inoculation of 106 parasitized red blood cells in a 0.1 ml carrier consisting of 47.5% Ringers (27 mM KCl, 27 mMCaCl2, 0.15 MNaCl), 50% heat-inactivated calf serum and 2.5% heparin (200 units mlK1). We infected mice in the P. chabaudi experiment with clone DK as this clone was used in Billingsley et al. (2005) and we used clone BS in the P. vinckei experiment.

Data collection –

Daily, following infection, we made thin blood smears from the tail vein to count asexuals, reticulocytes (immature red blood cells) and gametocytes and we measured red blood cell densities (anaemia) using flow cytometry (Coulter Electronics). We stained all smears for 15 min using 10% Giemsa buffered in 90% phosphate solution. We analysed blood smears at 1000! magnification without knowledge of treatment group. Following Crooks (2004), we counted at least 20 reticulocytes, 20 asexuals and 20 gametocytes.

However, we stopped counting if we had not reached these targets after searching 100 fields. We sexed P. vinckei gametocytes from smears, but P. chabaudi gametocytes are not readily sexed from smears using blood straight from the tail vein so we used the method described in Reece et al. (2003). As duration of patent parasitemia differs in our study species, we collected these data from days 2 to 15 and days 27 to

31 in the P. chabaudi experiment and days 3 to 8 in the P. vinckei experiment. Following Buckling et al. (1999b), we computed the proportion of asexuals that become gametocytes (gametocyte conversion rate).

Analyses -

Analyses were performed using SAS version 8.2 (SAS Institute 2002). We used repeated measures analyses to control for variation among mice and their infections. Repeated measures ANOVA is robust to deviations from multivariate normality and homogeneity of covariance matrices, although transformations to improve normality (evaluated using Shapiro–Wilk tests) nonetheless reduce the probability of obtaining a Type 1 error. Square root transformations were used on density estimates (for asexuals, gametocytes and reticulocytes). Sex ratios were arcsine square-foot transformed.

RESULTS

General observations -

Of 874 mosquitoes that we inspected at irregular intervals from both experiments, 23.8% had blood in their midgets that was visible under a dissecting scope. One control and one experimental mouse died in the first 3 days of the P. chabaudi experiment, possibly as a consequence of anaesthesia. A third experimental mouse developed a bruise on its tail at day 12, at which point we stopped collecting samples from it. Before Plasmodium vinckei infections were established, a single control mouse became ill from unknown causes and was removed from the experiment. Hence, sample sizes were less than 20 in both experiments.

P. chabaudi -

Estimates of asexual (rSZ0.83, p!0.0001) and gametocyte density (rSZ0.63, pZ0.004) for this species were highly repeatable between observers. Effects of P. chabaudi infections on red blood cell density were evident by day 2, but asexuals were not detectable in thin blood smears until day 5 and were at very low densities after day 11. Gametocytes were similarly not detected until day 5, but were still detectable at day 13.We thus confined analyses on asexuals to the period between days 5 and 11 and on gametocytes to days 5–13. Gametocyte conversion rates were calculated from day 5 to 11. No asexuals or gametocytes were found between days 27 and 31 in a subsample of smears from both control and probed mice, so we excluded these days from analysis. Sex ratios were only estimated from smears on days 6 through 9 and days 11 and 13. change significantly during the course of infection was mass (interaction between day and mass, Wilks' *l*Z0.1, F14,2Z0.9, pZ0.65); remaining infection parameters changed over the course of monitoring (p!0.05 in all cases).

P. vinckei -

Infection characteristics were similar to those reported elsewhere for P. vinckei (Carter & Walliker 1975; Reece et al. 2005). However, two control and two experimental mice did not develop patent parasitemias until most mice had passed peak parasitemias. Analyses including or excluding these mice gave quantitatively similar results, so we present data for all mice. Effects of infection on red blood cell densities were evident by day 3. Asexuals were detectable from day 2 to 10, whereas gametocytes were detectable from day 3 to 10, so we analysed data between these dates.

CONCLUSION

In this paper, we describe and characterize *PbCHT1*, the first chitinase gene isolated from a rodent malaria species. We show that the gene product, PbCHT1, contains putative proenzyme and chitin-binding domains and is orthologous to a previously described endochitinase, PgCHT1, from *P. gallinaceum*. Targeted disruption of the *PbCHT1* gene by double homologous recombination has allowed us to study the existence of other putative chitinase activities in *P. berghei* ookinetes as well as the role of PbCHT1 in mosquito infection in the absence and presence of a PM.

Our findings indicate that *P. berghei* ookinetes have a single chitinase activity because (i) no additional bands were recognized in Western blottings by a PgCHT1 active-site antibody, (ii) no specific products could be amplified from PbCHT1-KO ookinetes with *Plasmodium* chitinase gene-specific degenerate primers, and (iii) most importantly, no residual ookinete-derived chitinase activity was observed in PbCHT1-KO parasites. In fact, the same may be true for *P. falciparum* because only a single chitinase gene has thus far been described in the Malaria Genome Project databases, which now contain about 95% of the genome. Intuitively, if this assumption is correct and *P. gallinaceum* does indeed possess both types of chitinase genes, then it can be suggested that both *P. falciparum* and *P. berghei* share an avian *Plasmodium* ancestor and that each has retained a different one of the two chitinases.

Reductions in infectivity were observed both in the presence of a PM (gametocyte feeds) and in its absence (ookinete feeds). Although in the absence of complementation we cannot rule out the possibility that the reduced infectivity is the result of pleiotropic effects, the findings suggests that PbCHT1 plays a role other than PM disruption. We cannot rule out the possibilities that chitin is present in the microvillus-associated network or the epithelial cells themselves and that chitinase activity is required to allow ookinete egress from these tissues.

In this respect, it should be noted that chitin precursors are synthesized by epithelial cells and must traverse the microvillus-associated network to

form the mature PM. Experiments are in progress to investigate these hypotheses.

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