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Clonal diversity of a malaria parasite, *Plasmodium mexicanum*, and its transmission success from its vertebrate-to-insect host

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ABSTRACT

Infections of the lizard malaria parasite *Plasmodium mexicanum* are often genetically complex within their fence lizard host (*Sceloporus occidentalis*) harbouring two or more clones of parasite. The role of clonal diversity in transmission success was studied for *P. mexicanum* by feeding its sandfly vectors (*Lutzomyia vexator* and *Lutzomyia stewarti*) on experimentally infected lizards. Experimental infections consisted of one, two, three or more clones, assessed using three microsatellite markers. After 5 days, vectors were dissected to assess infection status, oocyst burden and genetic composition of the oocysts. A high proportion (92%) of sandflies became infected and carried high oocyst burdens (mean of 56 oocysts) with no influence of clonal diversity on these two measures of transmission success. Gametocytemia was positively correlated with transmission success and the more common vector (*L. vexator*) developed more oocysts on midguts. A high proportion (~74%) of all alleles detected in the lizard blood was found in infected vectors. The relative proportion of clones within mixed infections, determined by peak heights on pherograms produced by the genetic analyser instrument, was very similar for the lizard's blood and infections in the vectors. These results demonstrate that *P. mexicanum* achieves high transmission success, with most clones making the transition from vertebrate-to-insect host, and thus explains in part the high genetic diversity of the parasite among all hosts at the study site.

1. Introduction

The life cycle of malaria parasites (*Plasmodium* spp.) requires transfer of gametocyte cells from the blood of an infected vertebrate host (squamate reptile, bird or mammal) to a biting insect vector (sandfly or mosquito) (Fialho and Schall, 1995; Vickerman, 2005), where sexual recombination of the parasite occurs (Baton and Ranford-Cartwright, 2005). Transmission success of the parasite from vertebrate to vector is influenced by gametocyte density in the blood (Mackinnon and Read, 1999; Schall, 2000), sex ratio of male and female gametocytes (Robert et al., 1996), specific genotypes of the parasite clones replicating in the vertebrate host (de Roode et al., 2005a,b), and vector genotype (Beier, 1998). The genetic diversity of parasite clones within the vertebrate host could also be important for the transmission success of *Plasmodium*, both overall for an infection and for the individual genotypes in the infection, but such processes remain poorly understood (but see Taylor et al., 1997a,b; de Roode et al., 2005a,b).

Cross-sectional surveys of human malaria parasites, *Plasmodium falciparum, Plasmodium vivax* and *Plasmodium malariae*, (Anderson et al., 2000; Leclerc et al., 2002; Cui et al., 2003; Imwong et al., 2006; Bruce et al., 2007) and data on malaria parasites of non-human vertebrate hosts (Vardo and Schall, 2007) reveal that infected vertebrate hosts commonly harbour more than one genotype of parasite, and such multi-clonal infections are more common where transmission intensity is high. The high frequency of multi-clonal infections therefore suggests two questions regarding the role of clonal diversity for the overall transmission biology and population genetics of *Plasmodium*. Firstly, do all clones successfully make the transition from vertebrate-to-insect? The small amount of blood taken by a vector may not include mature gametocytes of all clones within that infection, and not all genotypes that are present in the blood meal may successfully mate and complete sporogony. Thus, there could be a bottleneck reducing clonal diversity during transmission. Chronic failure of all clones to make this transition into vectors could have broad consequences for the population genetics of *Plasmodium*. Second, does clonal diversity (single-clone versus mixed-clone) influence the overall transmission success of the parasite (de Roode et al., 2003, 2005b)? Transmission potential could be reduced within mixed-clone infections if overall gametocytemia for each genotype is reduced or if intraspecific competition for establishment within the vector exists (de Roode et al., 2003, 2005b). In contrast, overall transmission potential may increase if infection gametocytemia is higher for mixed-clone infections (Taylor et al., 1997a,b; Vardo-Zalik and Schall, 2009).

This study sought to determine how the genetic diversity of clones in the vertebrate host's blood influences transmission

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success of *Plasmodium mexicanum*, a parasite of fence lizards, *Sceloporus occidentalis*, and sandflies, *Lutzomyia vexator* and *Lutzomyia stewarti*, in northern California, USA (Fialho and Schall, 1995). The life cycle is similar to that of other *Plasmodium* species, with the exceptional use of a sandfly vector rather than a mosquito. Recently characterised microsatellite markers allow identification of parasite genotypes (Schall and Vardo, 2007; Vardo and Schall, 2007) and show that a high proportion of natural infections are multi-clonal (from 50% to 85% depending on which of the five annually sampled sites at the field station is considered). Experimental infections are readily established that vary in clonal diversity (Vardo-Zalik and Schall, 2008, 2009), and experimental feeds by sandflies produce oocysts that can be counted (Fialho and Schall, 1995; Schall, 2000) and genotyped (see Section 2, Section 2.2). Replicate experimental infections of *P. mexicanum* with known numbers of parasite genotypes in the natural vertebrate host species were established, and both overall transmission success and transmission success of individual clones into the sandfly vectors were measured. Transmission success of individual clones has been followed for *P. falciparum* in natural human infections (Huber et al., 1998) and experimental infections of the rodent malaria parasite *Plasmodium chabaudi* in laboratory mice (Taylor et al., 1997a,b), but to my knowledge this is the first experimental study to focus on genetic diversity and transmission success of a non-human malaria parasite in its natural vertebrate and insect hosts.

2. Materials and methods

2.1. Experimental infections and parasite genotyping

The study was conducted at the University of California Hopland Research and Extension Center, near the town of Hopland, Mendocino County, California, USA (Schall, 1996). Naturally infected lizards were captured during May 2005 from five annually sampled sites (Vardo-Zalik and Schall, 2008, 2009) to serve as blood donors to initiate experimental infections. Blood was obtained from a toe clip to make blood smears (treated with Giemsa's stain and examined at 1,000×) and dried blood dots on filter paper for molecular analysis. Infected lizards were identified by scanning blood smears. Donor infections were chosen that had high parasitemia of asexually replicating meront stages (> 25 meronts per 1,000 erythrocytes). In early May, few infections presented high meront densities, so of the sample of 312 lizards, only seven suitable donors were identified. *Sceloporus occidentalis* are small lizards so only a small number of recipient infections could be initiated from a single donor.

DNA from infected lizards was extracted using the Qiagen DNeasy extraction kit, following the manufacturer's protocol. The parasites from the donor lizards were genotyped for three microsatellite markers (Pmx306, 747 and 732) using PCR primers and conditions presented in Schall and Vardo (2007) (Table 1). Labeled PCR product was run on an ABI Prism genetic analyser and results analysed using GeneMapper software (ABI). Of the seven donors, only two contained a single-clone of the parasite (one allele per marker). Previous trials using six markers found that the three used here were efficient at revealing such single-clone infections. For the other donors, I estimated the clonal diversity of infections as the maximum number of alleles observed for any of the three microsatellite markers (two to four clones). This estimate is widely used and gives an unbiased estimate of the minimum number of clones in an infection (Anderson et al., 2000; Ferreira et al., 2002, 2007; Bogreau et al., 2006; Bruce et al., 2007; Vardo and Schall, 2007).

To create replicate recipient infections with known numbers of clones, 80 non-infected adult male lizards (snout-vent length >54 mm) were captured from two sites where malaria has been absent for the past 30 years (J. Schall, personal communication). Smears were examined at 1,000× to view at least 10,000 erythrocytes and a sensitive PCR-based protocol that detects extremely weak infections (as low as one parasite per 1 million erythrocytes) (Vardo et al., 2005) confirmed that the recipient lizards were not already infected with *P. mexicanum*. Treatment groups, each with 10 recipients, with differing clonalities (one, two or three or more clones [3+]) and genotype combinations (a, b, or c) were as follows: two single-clone groups (1a and 1b), three two-clone groups (2a–c), and three multi-clone groups (with three or more clones; 3–3c) (Table 2).

Infections were initiated following the protocol given in Vardo-Zalik and Schall (2008, 2009). Recipient lizards were infected with blood from one or two different donor infections (Tables 1 and 2). Briefly, blood was taken from each donor lizard and quickly mixed with PBS. Twenty microlitres of the blood–PBS mixture (containing approximately 200×10^3 asexual parasites) was injected i.p. into each recipient lizard. The number of parasites injected remained constant across all treatment groups regardless of clonality; for mixed-donor treatments, each donor contributed an equivalent number of parasites to the mixture, resulting in the final count of 200×10^3 asexual parasites in 20 µl of the blood–PBS mixture.

Lizards were housed by replicate in eight outdoor cages suspended from clotheslines and shaded under a tarp. Cages were moved weekly within the array. Lizards were fed daily to satiation on mealworms or crickets and on days where the ambient temperature exceeded 27 °C, were showered with cool water every 2 h from 1300 to 1900 h.

Table 1
Seven donor infections of *Plasmodium mexicanum* and their respective numbers of clones (one to four clones per infection). Alleles and clonality were assessed via three microsatellite loci (Pmx306, 747 and 732; Schall and Vardo, 2007). The minimum number of clones within an infection (estimated via the microsatellite locus with the most alleles for that infection) is presented. Alleles at each locus are presented in reference to repeat length, with the first letter/number representing the shortest repeat (306 A–H; 747 1–6; 732 a–f).

Donor	Number of Clones	Pmx306	Pmx747	Pmx732
I	1	H	4	c
II	1	G	4	c
III	2	A C	4	c f
IV	2	D F	6	d
V	2	D	4 5	a
VI	3	A D G	2 4	b c f
VII	4	B D E H	1 3	e f a

Table 2
Treatment groups for infections with differing clonalities and genotypes of the malaria parasite *Plasmodium mexicanum*. Treatments are represented by the number of clones (1–3, with 3 including infections with three or more clones of the parasite) and replicate treatment (a–c, each with differing genotype combinations). All groups have 10 individuals. Alleles and clonality were passed via three microsatellite loci (Pmx306, 747 and 732; Schall and Vardo, 2007). The minimum number of clones within an infection (estimated via the microsatellite locus with the most alleles for that infection) is presented. Alleles at each locus are presented in reference to repeat length, with the first letter/number representing the shortest repeat (306 A–H; 747 1–6; 732 a–f).

Treatment	Donor(s)	Number of clones	Pmx306	Pmx747	Pmx732
1a	I	1	H	4	c
1b	II	1	G	4	c
2a	III	2	A C	4	c f
2b	IV	2	D F	6	d
2c	V	2	D	4 5	c
3a	VI	3	A D G	2 4	b c f
3b	VI + VII	6	A D G B E H	2 4 1 3	b c f e a
3c	II + VII	5	B D E H G	1 3 4	c e f a