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J. Parasitol., 92(2), 2006, pp. 413–415
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Detection of a Malaria Parasite (*Plasmodium mexicanum*) in Ectoparasites (Mites and Ticks), and Possible Significance for Transmission

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ABSTRACT: Two species of sandflies (*Lutzomyia*) are competent vectors of *Plasmodium mexicanum*, a malaria parasite of lizards. The very patchy distribution of sites with high *P. mexicanum* prevalence in the lizards, and often low or even nil sandfly density at such sites, provoked an evaluation of 2 common lizard ectoparasites, the tick *Ixodes pacificus* and the mite *Geckobiella occidentalis*, as potential passive vectors. *Plasmodium* sp.-specific polymerase chain primers were used to amplify a long segment of the mitochondrial cytochrome *b* gene that is unlikely to survive intact if the parasite cells are killed within a blood-feeding arthropod. The segment was strongly amplified from sandflies (the positive control for the method) from 1 to 96 hr postfeeding on an infected lizard. For ticks, the gene fragment was poorly amplified at 0 hr postfeed, and not amplified after 2 hr. In contrast, strong amplification of the parasite DNA was observed from mites from 0 to 20 hr postfeed, and weak amplification even at 96 hr.

Plasmodium mexicanum, a malaria parasite of western fence lizards (*Sceloporus occidentalis*) in California, is the only known species of *Plasmodium* that is transmitted by a vector other than a mosquito. Compelling evidence indicates that 2 species of sandflies (*Lutzomyia vexator* and *Lutzomyia stewarti*) are competent vectors for the parasite. First, the parasite completes normal development in the sandflies, producing mature oocysts on the midgut and sporozoites that travel to the salivary glands (Ayala and Lee, 1970; Fialho and Schall, 1995). Second, laboratory experiments successfully transmit the parasite by injection of sporozoites into uninfected lizards (Ayala, 1971; Fialho and Schall, 1995) or allow infected sandflies to take a blood meal on an uninfected lizard (Klein et al., 1987). Last, the parasite has evolved a mechanism to manipulate the sandfly's thermoregulatory behavior to raise the vector's temperature to one that is optimal for the development of *P. mexicanum* (Fialho and Schall, 1995), indicating a close association of the sandflies and parasite.

Despite this evidence, the transmission ecology of the parasite remains an open and vexing question. At a long-term study site in northern California (Schall, 2002a), prevalence of the parasite can be high in its lizard host, with up to 50% of lizards infected at some local areas. The vectors, though, are not abundant, and in some areas where the parasite prevalence is high, vectors are not detectable using standard survey methods (Schall and Marghoob, 1995; J. Schall and T. Smith, pers. obs.). Most problematic is the very patchy distribution of high versus low prevalence areas. Eisen and Wright (2001) found over a 3-yr period that high prevalence patches of only a few hundred square meters may be surrounded by areas with substantially lower prevalence, with groundcover being a strong predictor of prevalence. These results hint that there may be other vectors of *P. mexicanum*. For example, the

very local distribution of the parasite matches trends in density of ticks and mites at the Hopland site (Talleklint-Eisen and Lane, 1999; Schall et al., 2000).

Because the sandflies are the only known biting insects to feed on fence lizards at the site, we chose to focus on 2 common ectoparasites of the lizards, the mite *Geckobiella occidentalis* and the tick *Ixodes pacificus* (Schall et al., 2000). Another apicomplexan parasite of the lizards, *Schellackia occidentalis*, is transmitted by the mite and prevalence of this parasite is higher in the fence lizards than *P. mexicanum* (data not shown). *Schellackia occidentalis* does not replicate or undergo development within the mite, but is passively transmitted when a lizard eats a mite that has very recently fed on an infected lizard (Bonorris and Ball, 1955; Klein et al., 1988). We regarded the ectoparasites as unlikely passive vectors of *P. mexicanum*, and thus sought a rapid method to eliminate them from further consideration.

We used a technique to determine whether *P. mexicanum* can be detected in the ectoparasites for periods from 0 to 96 hr after they ceased feeding on the blood of infected lizards. Polymerase chain reaction (PCR) primers that are specific to species of *Plasmodium* and related parasites, but will not anneal to any region of the arthropod genome (or that of the lizard or of *Schellackia occidentalis*), were used to amplify a moderately long DNA segment of the cytochrome *b* gene (1,174 base pairs [bp]). The parasite must survive in a competent passive vector, and thus the long DNA segment would remain intact, but in an ectoparasite that is not a suitable passive vector, the parasite would be killed when the blood meal is digested and the long segment would most likely not survive. Studies on the survival of long DNA segments from a parasite in known unsuitable versus competent vectors are not available for comparison. However, in support of the method used here, many studies have detected DNA from the blood of the vertebrate in blood-feeding arthropods. Only short DNA segments from a target gene (for example, 300 bp for the cytochrome *b* gene) can be detected (Lee et al., 2002; Malmqvist et al., 2004), and quality of the DNA drops sharply within a few hours during digestion by the arthropod (Chow-Shaffer et al., 2000; Lee et al., 2002; Mukabana et al., 2002).

The study was conducted at the University of California Hopland Research and Extension Center, a 2,169 ha property near the town of Hopland in southern Mendocino County, California. A study on this parasite-host system has been underway since 1978 (Schall, 1996; 2002a). Lizards were collected and blood smears made to be stained with Giemsa. Examination under $\times 1,000$ allowed choosing of infected and uninfected lizards for the study; very weak infections, not detectable under the microscope, are rare at Hopland (Perkins et al., 1998).

We trapped sandflies from rodent (*Citellus beecheyi*) burrows; previously fed females are rarely encountered in the traps (Schall, 2002b).

TABLE I. Number of sampled sandflies, ticks, and mites that produced PCR product (band apparent on gel) for a 1,174-bp fragment of the cytochrome *b* gene of *Plasmodium mexicanum*.

Hours postfeed*	Sandflies†	Ticks†	Mites†
0		8/16	18/18
1	6/6	5/6	1/1
2	8/8	2/4	12/14
5	7/7	0/8	15/16
10	6/6	0/4	16/17
20			1/1
96	6/6		7/15

* Hours since the sandfly or ectoparasite last fed on an infected lizard.

† Number producing bands/total number sampled.

Sandflies and a lizard (either infected or not infected with *P. mexicanum*) were placed into a cloth cage (20-cm cube) and kept at room temperature. Blood-engorged female sandflies were removed and transferred to an 18-ml plastic vial containing a layer of slightly moistened plaster of Paris in its base, and a small piece of fructose-soaked cotton was placed on the screen top of each vial for the insects to feed. We placed the vials into an incubator set at 26 C, the optimal temperature for parasite development (Fialho and Schall, 1995). Sandflies that had fed on an infected lizard were placed into 100% ethanol at 1, 2, 5, 10, or 96 hr postfeed. These sandflies provided a positive control for the method of detecting the parasite. Sandflies that had fed on an uninfected lizard were placed into alcohol at 0 hr postfeed. Lizards naturally infested with mites or ticks were inspected under a dissecting microscope and the ectoparasites were removed to be placed into 18-ml vials with the moist substrate and folded pieces of paper to provide climbing surfaces. The vials were stored at 26 C. Ticks were placed into 100% ethanol from 0, 1, 2, 5, and 10 hr after being removed from the lizard (thus, postfeed), and the mites from 0, 1, 2, 5, 10, 20, and 96 hr. The alcohol in each vial was changed twice in the next 24 hr, and the vials then stored at 4 C.

DNA from the arthropods was extracted using a DNeasy kit (Qiagen, Valencia, California). A single sandfly, mite, or tick was placed into a 1.5-ml vial, allowed to dry for several minutes, and then crushed with a plastic pestle (Sigma, St. Louis, Missouri) in tissue lysis buffer from the kit. The pestle was discarded after a single use. The target 1,174-bp fragment of the *P. mexicanum* mitochondrial cytochrome *b* gene was amplified with the primers DW2 and DW4 (Perkins and Schall, 2002): DW2 forward, 5'-TAA TGC CTA GAC GTA TTC CTG ATT ATC CAG-3'; and DW4 reverse, 5'-TGT TTG CTT GGG AGC TGT AAT CAT AAT GTG-3'. PCR was performed using Ready-to-Go beads (Amersham, Piscataway, New Jersey) in a 25- μ l reaction. Added to the bead in a 0.5-ml vial were 22 μ l of water, 2 μ l of template DNA, and 1 μ l of each 10 μ M primer. The PCR program used was 94 C for 4 min, followed by 35 cycles of 94 C for 20 sec, 60 C for 20 sec, and 72 C for 90 sec, followed by a final 7-min extension at 72 C. A positive control using template DNA from a known infected lizard's blood was included in each PCR procedure. Two kinds of negative controls were used (i.e., water replacing template DNA and extracted DNA from a sandfly or ectoparasite that had fed on an uninfected lizard). The latter control assured that the primers were amplifying only parasite DNA. PCR product was visualized on 1% agarose gel treated with ethidium bromide. Some PCR products were cleaned through Centricon columns (Princeton Separations, Adelphia, New Jersey) and subjected to cycle sequencing with BigDye terminator mix (ABI, Foster City, California) using the DW2 forward primer, and run on the ABI Prism automated sequencer.

No PCR product (i.e., no band on the gel) was detected for any of the arthropods that had fed on an uninfected lizard, confirming the specificity of the primers for the parasite. PCR product from the sandfly samples always presented a bright band on the gel from 1 to 96 hr postfeed (Table I, Fig. 1A). The PCR product from 2 sandflies, from 10 and 96 hr postfeed, was sequenced; sequences were identical to those reported for *P. mexicanum* (Perkins and Schall, 2002). In contrast, only

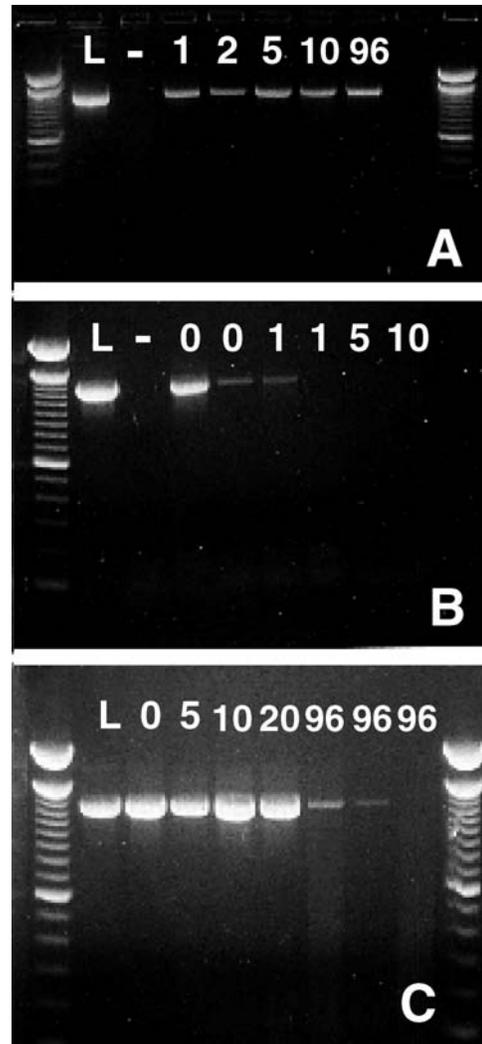


FIGURE 1. Agarose 1% gel stained with ethidium bromide, showing PCR product of an amplified 1,174-bp fragment of the cytochrome *b* gene of *Plasmodium mexicanum*. Parasite DNA was extracted from three blood-feeding arthropods, sandflies (*Lutzomyia* spp.), ticks (*Ixodes pacificus*), and mites (*Geckobiella occidentalis*) that had fed on an infected fence lizard (*Sceloporus occidentalis*). A reference positive control was DNA extracted from the blood of an infected lizard. Negative controls shown are result of PCR using template DNA from a sandfly and tick fed on an uninfected lizard; not shown is the similar negative control from a mite fed on an uninfected lizard included for each PCR run. Hours postfeeding when the ectoparasite was preserved are given (see text). Results for sandflies (A), ticks (B), and mites (C).

half the ticks taken at 0 hr postfeed revealed a positive result, and none were positive by 5 hr postfeed (Table I, Fig. 1B). Of the ticks presenting a positive result, 7 of 15 were faint bands (Fig. 1B), most likely indicating that very little intact template DNA was present in the extraction. A contrasting result emerged for the sampled mites. Fully 94% of 67 mites were positive from 0 to 20 hr postfeed, most with bright bands (only 3 of 67 presented faint bands on the gels). Only half of 15 mites sampled at 96 hr gave a positive result, and all of these bands were faint (Table I, Fig. 1C). PCR product from 2 mites sampled at 10 hr postfeed was sequenced with results identical to the known *P. mexicanum* sequence.

P. mexicanum experiences starkly different fates within each of the blood-feeding arthropods. In sandflies, gametocytes of *P. mexicanum* emerge from their host cell within minutes after the blood leaves the lizard (Osgood and Schall, 2003), undergo mating, and the parasite

reaches maturity on the vector midgut in 6 days at 26 C (Fialho and Schall, 1995). Thus, the parasite survives the digestive processes of the sandfly. Every sandfly tested here revealed a strong positive PCR amplification of the target DNA segment, from 0 to 96 hr after feeding on an infected lizard. These results provide a positive control for the assay method. In ticks, the parasite appears to be killed quickly because the DNA segment was degraded and often failed to amplify even immediately after the feeding tick was removed. In contrast, the long DNA segment was strongly amplified from mites for at least 20 hr after feeding, and half were weakly amplified fully 96 hr after feeding ended.

In ixodid ticks, feeding and digestion is simultaneous, and destruction of the host blood cells occurs immediately after entering the tick, whereas digestion of blood by mites takes longer and requires construction of a peritrophic membrane around the blood meal (Mehlhorn, 2000). Thus, the more leisurely digestion of blood by *G. occidentalis* may allow the parasite to remain alive for at least 20 hr.

The behavior of *G. occidentalis* under natural conditions is unknown, but we have observed fence lizards in cages, and mites very often leave the lizard hosts. Uninfested lizards placed into the cages with lizards carrying the mites are very quickly infested. We have not seen lizards eating the mites, in agreement with observations reported by Klein et al. (1988). However, fence lizards at the Hopland site must often eat mites because *Schellackia occidentalis* infection is common in the lizard population and is transmitted when recently fed mites are ingested by a lizard.

Although a mite passive vector would explain the very fine-scale prevalence patterns of *P. mexicanum* in fence lizards, the lack of digestion of long DNA segments over more than 20 hr in the mite's blood meal, however suggestive, does not demonstrate the parasite cells are alive or able to be transmitted passively if a lizard eats a mite carrying infected blood. We did not expect the results presented here for the mites, and thus did not conduct transmission experiments. Indeed, the purpose of the study was to quickly discount the ectoparasites as possible passive vectors. Therefore, transmission experiments are pending. The mite's digestive tract must be a hostile environment for a *Plasmodium* sp. cell, both because of the presence of lytic enzymes and a steep drop in oxygen concentration. However, sporozoites of *Schellackia occidentalis* survive the gut of the mite and later, consumption by lizards. Avian malaria parasites can survive in blood kept at surprisingly high temperatures within capillary tubes (Caldwell, 1944), and this ability was found in similar experiments with rodent and lizard malaria parasites (Schall, 1990; data not shown), demonstrating that *Plasmodium* sp. parasites are not fragile organisms.

We thank S. M. Osgood for advice and assistance in both the field and laboratory, and E. Glesmann for her help in capturing and maintaining lizards. The staff of the Hopland Research and Extension Center offered significant logistical support, and C. Vaughn allowed us to use a portion of his research laboratory at the field station. The research was funded by a grant from the United States National Science Foundation.

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