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PCR Detection of Lizard Malaria Parasites: Prevalence of *Plasmodium* Infections with Low-Level Parasitemia Differs by Site and Season

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ABSTRACT: *Plasmodium*-specific polymerase chain reaction (PCR) primers allowed detection of infections with very low-level parasitemia for 3 species of malaria parasites infecting *Anolis* lizards at 2 Caribbean sites, Puerto Rico and Saba, Netherlands Antilles. A verification study, using a single-tube nested PCR to eliminate contamination, showed that infections as low as 1 parasite per millions of erythrocytes could be detected by amplifying a 673 bp fragment of the cytochrome b gene. Very low-level parasitemia infections, subpatent under the microscope, were common in *A. sabanus* on Saba sites, with no significant seasonal difference (31% of infections appearing uninfected by microscopic examination in summer were found infected by PCR, 38% in winter). At the Puerto Rico site, the subpatent infections were also common in *A. gundlachi*, but were more prevalent in winter (53%) than in summer (17%). A similar high frequency of subpatent infections is known from studies on human and bird malaria, but a previous PCR-based study on a temperate lizard malaria system found few such low-level infections. Differences in the prevalence of subpatent infections by site and season suggest transmission biology may select for distinct life history strategies by the parasite.

The prevalence of malaria parasites (*Plasmodium*) in their vertebrate host populations has traditionally been determined by microscopic examination of blood smears. Parasitologists have long known that this is an inaccurate method because many infections are in the subpatent phase with low-level parasitemia, resulting in these infections being falsely scored as negatives. Examination of multiple smears taken over time from suspected infected hosts is the classic method to correct for this error (Macdonald, 1926; Herman, 1938). Two modern techniques, polymerase chain reaction (PCR) amplification of parasite DNA and serological tests, allow a more accurate estimate of infection prevalence. The PCR detects extremely low-level infections in the blood, far below the parasitemia needed for success, even if multiple slides are scanned (Perkins et al., 1998; Richard et al., 2002; Hellgren et al., 2004). However, PCR will fail to detect infections located only in deep tissues or later infections that have ceased parasite replication in the blood. Serological techniques are more precise in such cases (Jarvi et al., 2002), but may produce false positives for infections that have been cleared from the host.

Although PCR provides an imperfect estimate of the actual prevalence of malaria parasites, the technique may allow insight into the life history ecology of *Plasmodium* spp. For example, previous PCR studies demonstrated that the proportion of false negatives (negative after scanning smears, but positive by PCR) can be high (up to 67% for *Plasmodium* spp. infections of humans) but lower in birds (about 25% of birds sampled) (reviewed in Perkins et al., 1998; Richard et al., 2002), and very low in a study of the lizard malaria parasite, *P. mexicanum*, at a California site (only 4–6% of the samples were false negatives) (Perkins et al., 1998). The lizard malaria study in California also found differences in the prevalence of subpatent infections by site, with a high proportion of all infections being low-level at a site with low overall prevalence, and rare at a site where overall prevalence was high. Thus, very low-level parasitemia infections were far more common where transmission may be less intense. This suggests that such infections may be a life history strategy by the parasite when faced with times or places with rare or even impossible transmission, and in such cases the parasite may reduce its rate of replication or parasitemia to insure minimal cost to its host (Gill and Mock, 1985; Ewald, 1994).

Here, we report the proportion of subpatent infections detected by PCR for lizard malaria parasites at 2 tropical sites, a rain forest in eastern Puerto Rico and on the tiny island of Saba, Netherlands Antilles. *Anolis gundlachi* in Puerto Rico and *A. sabanus* on Saba are infected

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with the same 3 species of *Plasmodium*: *P. floridense* and 2 species now combined under *P. azurophilum* (Staats and Schall, 1996; Schall et al., 2000). The *P. azurophilum* complex includes 2 morphologically identical parasites, one infecting erythrocytes, and the other infecting several classes of white blood cells (Perkins, 2000; 2001).

Prevalence is slightly higher in Puerto Rico compared to the California lizard malaria system (20-45% among sites and males vs. females in Puerto Rico), and substantially higher on Saba (50-70% at sites used in this study) (Staats and Schall, 1996; Schall et al., 2000). Examination of blood smears of samples taken over the period 1992–2002 on Saba revealed no seasonal variation in prevalence of the parasites. However, apparent prevalence between 1996 and 2002 at the Puerto Rico site was consistently lower in the winter compared to in the summer (Staats and Schall, 1996; Schall et al, 2000; data not shown). This change in apparent prevalence was observed even for samples taken 5 mo apart, when the actual proportion of lizards infected was unlikely to have changed.

These results suggest that the ecology of the parasite-host association, including transmission biology, differs among the 2 tropical sites and the temperate California system. If differing transmission ecology selects for distinct life-history strategies by the parasites, the occurrence of very low-level infections should differ among sites and seasons in the Caribbean even though the same 3 parasite species are present. We use the results of a PCR study to test this prediction.

The study sites were the El Verde Field Station within the Luquillo Mountains in northeastern Puerto Rico (Waide and Reagan, 1996; Schall et al., 2000) and several sites near the town of The Bottom on Saba, Netherlands Antilles (Staats and Schall, 1996). Lizards were collected by hand or slip noose on a fishing pole, and a blood sample taken from a toe clip. Blood drops were used to make thin smears (to be stained with Giemsa) and dried dots on filter paper. Samples used in this study were taken from 1996 to 1999 on Puerto Rico and from 1997 to 1999 on Saba.

DNA was extracted from dried blood dots using the DNeasy kit (Qiagen, Valencia, California) and the provided protocol, except only 30 μl of elution buffer was used during the final stage. PCR primers specific for the *Plasmodium* cytochrome b gene were used in a nested set of reactions. An outer reaction used primers DW2 (5′ TAA TGC CTA GAC GTA TTC CTG ATT ATC CAG 3′) and DW4 (5′ TGT TTG CTT GGG AGC TGT AAT CAT AAT GTG 3′) with 1 μl of each 10 μM primer and 2 μl of extracted DNA in a 25 μl PCR reaction using Ready-to-Go beads containing dNTP, *taq* polymerase, and buffers (Amersham, Piscataway, New Jersey). Reaction conditions were a preliminary step of 94 C for 4 min, followed by 35 cycles of 94 C for 20 sec, 60 C for 20 sec, 72 C for 90 sec, with a final step of 72 C for 7 min. A second reaction used 1 μl of the first PCR product and primers DW1 (5′ TCA ACA ATG ACT TTA TTT GG 3′) and DW3 (5′ TGC TGT ATC ATA CCC TAA AG 3′), and reaction conditions of a preliminary step of 94 C for 1 min, followed by 40 cycles of 94 C for 20 sec, 50 C for 20 sec, 72 C for 90 sec, with a final step of 72 C for 7 min. Extreme care was taken to prevent contamination during the second reaction, and a control known positive and negative (water replacing DNA) was included in each set of reactions. Presence of the 673 bp product from the second reaction was visualized on a 1% agarose gel. The primers amplify DNA from all 3 species of *Plasmodium* found at the 2 sites. This was determined by amplifying solitary infections and sequencing the DNA fragment (Perkins, 2000, 2001).

Two kinds of verification runs were performed to determine the sensitivity of the PCR technique. First, known infected lizards (known positives) were chosen and their parasitemia determined by counting parasites seen in 1,000 erythrocytes. These samples were then assayed using the above nested PCR reactions. A second verification method eliminated an important source of contamination during nested PCR. Preliminary results revealed an apparent high proportion of false negatives in the sampled lizards, so we wished to eliminate the possibility that contamination during the transfer of PCR product from the first reaction to the next PCR was presenting spurious results. Therefore, we extracted DNA from a known infected lizard, then diluted the DNA to determine the lowest concentration when the nested method detected the parasite. Contamination was prevented during this procedure by using a modification of the single-tube nested PCR method of Abath et al. (2002). Briefly, each inner primer (DW1 and DW3) was diluted to 1 μM, and 10 μl of each primer was put inside the cap of a 0.5 ml vial

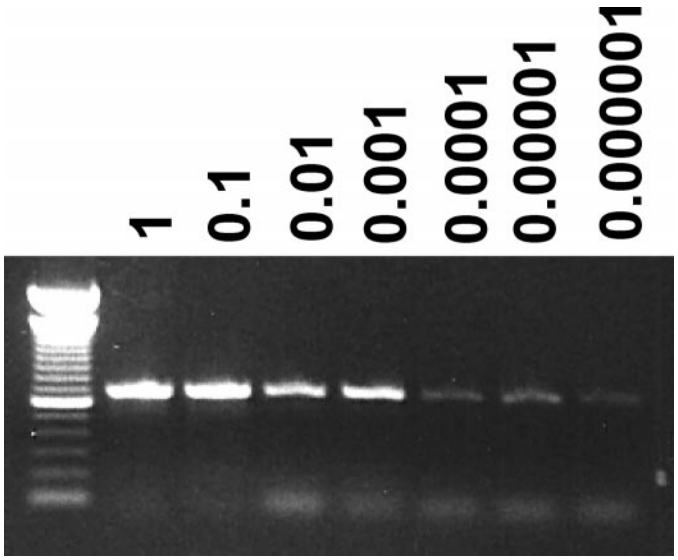

FIGURE 1. 1% agarose gel showing PCR product obtained by single-tube nested PCR for a 673 bp segment of the cytochrome b gene of *Plasmodium*. DNA was extracted from an infected lizard, then diluted from ×1 to ×0.000001. Bands are seen for all dilutions, but weak bands for 0.0001 or lower concentration of the extracted DNA.

and allowed to dry. The 2 outer primers (DW2 and DW4) were diluted to 0.01 μM concentration. A PCR mixture was prepared as described above for an outer reaction, then 25 μl gently pipetted into the vial with the dried inner primers on its lid, and 20 μl of oil was placed on the surface of the reaction mixture. The vial was closed and placed into a PCR thermalcycler with its heated lid turned off. The outer PCR reaction was used, but with only 10 cycles. The reaction mixture was allowed to cool to 15 C, and then the vial was shaken vigorously to mix the inner primers into the mixture. The second PCR (inner reaction conditions) was then run. The low concentration of outer primers allowed them to be completely consumed in the first reaction. Again, the PCR product was visualized on a 1% agarose gel. The success of the single tube PCR method in detecting extremely low concentrations of parasite DNA argued that the high proportion of false negatives discovered was not a result of contamination. We, therefore, reverted to the 2-tube method for the study, but used extreme caution when opening tubes and set up reactions under a UV hood to destroy errant DNA.

Blood samples from lizards scored as negative after a 6 min scan of blood smears under ×1,000 magnification were used for template DNA in the nested PCR protocol. Any samples showing a positive PCR reaction (thus a false negative under the microscope) led to a second examination of the blood smear to determine if any errors were made when scanning slides. A few such cases were found, and these samples were placed into the known positive data category. That is, the study's purpose was not to determine the accuracy of the slide scanning, but to find low-level infections that were not obvious under the microscope.

All 83 known positives (38 from Saba and 45 from Puerto Rico) produced product readily observable on the gel. Of these, 50 were from infections with parasitemia from <1 to 5 parasites per 10,000 erythrocytes (32 *P. azurophilum* [erythrocyte-infecting species], 6 *P. azurophilum* [species in white blood cells], 7 *P. floridense*, and 5 that could not be identified to species). An infection with parasitemia of 314/10,000 erythrocytes was diluted from ×1 to ×0.000001 for use in the single-tube nested PCR. All dilutions produced a band on the agarose gel, although a bright band was seen only to a dilution of ×0.001 (Fig. 1). Thus, an infection with parasitemia of 1 parasite per 30×10^6 erythrocytes can be detected by the assay.

False negatives (negative by slide scanning but positive by the PCR assay) were common for both Puerto Rico and Saba. On Saba, 38% (N = 40) of winter samples and 31% (N = 48) of summer samples proved to be false negatives, with no difference between winter and summer ($\chi^2 = 0.379$, $P = 0.538$). On Puerto Rico, false negatives were more common in winter than summer, 53% (N = 30) in winter and 17% (N

= 46) in summer ($\chi^2 = 10.85$, $P = 0.001$). There was no difference in proportion of false negatives for the summer samples comparing the islands ($\chi^2 = 1.174$, $P = 0.187$), but differed for the winter samples ($\chi^2 = 3.93$, $P = 0.047$).

The PCR method proved a highly sensitive assay for very low-level parasitemia infections. The dilution trial demonstrated that an infection can be detected with parasitemia of 1 parasite per millions of red blood cells. Our 6 min scanning protocol allows examination of approximately 10,000 cells. To detect such a very low-level infection would require scanning multiple smears for 300 hr. Richard et al. (2002) similarly found that a nested PCR for cytochrome b was very sensitive for detection of avian malaria infections.

Very low-level parasitemia infections were common on both Caribbean islands, similar to the reported results for human and bird malaria, but subpatent infections were far more common than found in the study of a temperate lizard malaria system. False negatives after microscope scanning were only 4–6% of samples in California, but 17–53% for the 2 tropical locations. This overall lower parasitemia for the Caribbean lizard malaria parasites may account for the lack of virulence observed for these species compared to the high virulence seen for *P. mexicanum* infections (Schall and Pearson, 2000; Schall, 2002; Schall and Staats, 2002).

Infections at the earliest stages, when parasites have just entered the blood, would be subpatent under the microscope. Some of the infections found by PCR on the tropical islands likely fell into this category, but we frequently recapture infected lizards on both Puerto Rico and Saba after several months or a year, so it is highly unlikely that the 2 Caribbean sites experience a turnover of infections high enough to account for the high proportion of very weak parasitemia infections observed there. A more likely explanation is that the parasite follows a life-history strategy that keeps replication and parasitemia low for some sites and seasons. Transmission at the California site is strongly seasonal, and parasitemia drops during the winter when lizards are dormant only to rebound again the next spring (Bromwich and Schall, 1986; Eisen, 2000). High parasitemia may be optimal for infections during the short transmission period, especially if many infections contain competing clones of parasites. In contrast, lizards are active year-round at the tropical sites and transmission may be far less seasonal on the islands. However, the sharp increase in low-level infections during winter in Puerto Rico suggests that transmission there may be reduced during that period.

One other important difference between the temperate and tropical study systems is the presence of 3 *Plasmodium* species in the Caribbean, and only 1 in California. There is no way at this time to determine if subpatent infections are more common for 1 or 2 of the 3 species on Puerto Rico or Saba. That is, the positive result on the PCR analysis does not provide information about which species was or were being detected. Theoretical discussions of the life-history strategies of parasites typically revolve around transmission biology, such as seasonality or vector abundance, with less discussion on the genetic structure of the infections (but see Eisen and Schall, 2000). The role of interspecific interactions of malaria parasites on infections is even more rarely examined (Schall and Bromwich, 1994). The role of interspecific interactions among species of Caribbean lizard malaria remains an open question. For example, the 3 *Plasmodium* species infecting *Anolis* spp. on Caribbean islands may well drop to low levels as they compete within an individual host, perhaps through manipulation of the host immune system.

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