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Use of PCR for detection of subpatent infections of lizard malaria: implications for epizootiology

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Abstract

The estimated prevalence of a malaria parasite, *Plasmodium mexicanum*, of western fence lizards, *Sceloporus occidentalis*, was compared using two techniques: microscopic examination of blood smears, and nested PCR amplification of the 18S small subunit rRNA gene. Two sites in northern California, USA were investigated, one with known long-term high prevalence of the parasite (30% by blood smear scanning), and one with low prevalence (6%). The nested PCR readily detected very low-level infections (< 1 parasite per 10 000 erythrocytes); such infections are often subpatent by normal microscopic examination. False negatives (scored as not infected after scanning the blood smear, but found infected via PCR) were rare at both sites (4% at the high-prevalence site, 6% at the low-prevalence site). However, a greater proportion of infections was detected only by PCR at the low-prevalence site (50% vs. 9%). If 50% of the infections sustain very weak parasitaemia where lizards are rarely infected, this would accord with hypotheses that predict that parasites should reduce infection growth when transmission is uncommon. The study demonstrates that PCR is a powerful tool to detect very low-level malarial infections in vertebrate hosts, including those with nucleated erythrocytes.

Keywords: epizootiology, lizard, malaria, PCR, *Plasmodium*, *Sceloporus*

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Introduction

A study on the ecology of any species begins with information on its abundance. For parasites, abundance is measured as prevalence (percentage of suitable hosts infected) and parasitaemia (number of parasites infecting an individual host). Accurate measures of prevalence and parasitaemia are required for applied parasitological studies such as epidemiology and public health efforts to control diseases of human or veterinary importance. Additionally, detection of infections with the lowest level parasitaemia is critical for testing hypotheses on the evolution of life-history traits and virulence. For example, Gill & Mock (1985) and Ewald (1994) proposed that during periods of rare or impossible transmission, selection would favour a reduction in parasitaemia to decrease parasite-induced mortality in the host. If infections with low parasitaemia are often missed in studies, this would seriously compromise tests of the hypothesis by biasing estimates of prevalence toward the more severe, easily detected infections.

The prevalence of malaria parasites (*Plasmodium* spp.) in their vertebrate hosts has traditionally been determined by scanning stained blood smears. However, researchers have known for a considerable time that this method frequently misses infections of very weak parasitaemia (Macdonald 1926). Recent work using the polymerase chain reaction (PCR) and *Plasmodium*-specific primers have allowed more accurate detection of the parasite when in low densities in the blood, including infections in human hosts that are subpatent by microscopic examination (Sethabutr *et al*. 1992; Snounou *et al*. 1993; Wataya *et al*. 1993; Bottius *et al*. 1996). Molecular techniques to screen for *Plasmodium* infections have only been used for one nonhuman host. In a study of Hawai'ian birds, Feldman *et al*. (1995) found that quantitative competitive PCR could detect as few as eight parasites per 10 000 host red blood cells. This proved to be a particularly sensitive assay, as DNA extracted from avian blood may contain primarily host DNA from the nucleated erythrocytes which could have reduced the efficiency of PCR amplification of the target parasite gene.

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Here we present a comparison of thin blood film vs. PCR determination of infection status for the malaria parasite *Plasmodium mexicanum* of the western fence lizard *Sceloporus occidentalis* at two sites in northern California, USA. We chose this system because it has been under study for 20 years and has provided perhaps the best indication of a cycle in the prevalence of *Plasmodium* in its vertebrate host (Schall & Marghoob 1995; Schall 1996). The apparent cycle could indicate real changes in the prevalence of the parasite, but could also be driven by changes in parasitaemia causing many low-level infections to remain undetected in some years.

Our goals were: (i) to assay the ability of a nested PCR of the 18S small subunit rRNA gene to detect very low parasitaemia of *P. mexicanum* in the nucleated erythrocytes of lizards; (ii) to compare prevalence of the parasite determined by slide scanning and the PCR technique; and (iii) to compare the abundance of infections with very low parasitaemia (subpatent by microscopic examination) at a site with low prevalence (presumed low transmission intensity) to a site with high prevalence (high transmission). Ewald (1994) suggests that rare transmission should select for parasites with reduced virulence resulting in low reproductive rates and parasitaemia.

Materials and methods

Lizards were collected at the University of California Hopland Field Station in southern Mendocino County, California, USA from two sites 4 km apart, one with a long-term high prevalence (30% in 1996; $N = 313$) and one with low prevalence (6% in 1996; $N = 139$). Blood was extracted from each lizard via a toe clip; 2–3 drops were placed on filter paper, dried, and stored in a plastic bag containing silica gel. Another drop was used to make a thin smear. Smears were stained with Giemsa and scanned at 1000× for 6 min which allows examination of ≈ 10 000 erythrocytes. This has been the standard method used for 20 years at Hopland (Schall 1996).

Total DNA was extracted from the dried blood samples using proteinase K digestion in 400 μL of stain extraction buffer (0.01 M Tris, 0.01 M Na_2EDTA, 0.1 M NaCl, 0.07 M SDS, at pH 8.0, and 0.006 g DDT per mL of buffer added at the time of extraction), phenol–chloroform–isoamyl alcohol separation of proteins, and cold ethanol precipitation. Hydrogen peroxide (3%, 25 μL) was added to the initial digestion to degrade haeme which can decrease PCR efficiency (Akane 1996).

The 18S rRNA gene was assayed with primers from Li *et al*. (1995): (570) 5′-CGACTTCTCCTTCCTTTAAAAGATAGG-3′ and (566) (5′-GGATAACTACGGAAAAGCTGTAG C-3′), in a 25-μL reaction mixture containing 1 unit of *Taq* polymerase, 2.5 mM $MgCl_2$, 200 μM of each dNTP, and 2.5 μL of Perkin-Elmer GeneAmp 10× PCR buffer (100 mM Tris-HCl, 500 mM KCl, and 0.01% gelatin). For some reactions, Ready-To-Go™ PCR beads (Pharmacia BioTech) were employed. Each of these contains 1.5 units of *Taq* polymerase, 10 mM Tris-HCl, 50 mM KCl, 200 μM of each dNTP and stabilizers. The reaction mixture was heated to 95 °C for 5 min and then amplification was performed through 35 cycles at 95 °C for 1 min, 48 °C for 1 min and 72 °C for 2 min. The nested PCR reaction used 1 μL of the initial reaction as a template with primers (841) 5′-GAACGAGATCTTAACCTGC-3′ and (844) 5′-TATTGATAAAGATTACCTA-3′ and the same amplification conditions except that the annealing temperature was raised to 50 °C. PCR product was electrophoresed on a 2% agarose gel in 1× Tris–borate (TBE) buffer stained with ethidium bromide. The detection of a band of the appropriate size under UV light was scored as positive for *Plasmodium*. Both sets of primers are specific to *Plasmodium*; noninfected lizards and lizards infected with other blood parasites (e.g. haemogregarines) showed no amplification.

To verify the ability of PCR to detect malaria parasites, a range of parasitaemia was first tested (53 known infected samples from the high-prevalence and six from the low-prevalence site). Some of these were part of a mark–release program where infections were followed over time and occasionally provided smears that required extended scanning to detect the first parasite, or were apparently negative even after such detailed study. These infections provided the lowest parasitaemia of known infected lizards (< 1 parasite/10 000 red blood cells). The parasitaemia of infections used in the verification study were: < 1/10,000–1/1000 erythrocytes (eight infections); 2–5/1000 (18 infections); 6–40/1000 (17 infections); and > 40/1000 (16 infections).

A random sample of 159 lizards scored as noninfected by slide scanning was used in the PCR-based search for false negatives. Each set of PCR reactions contained a known positive control, typically a weak infection (one parasite/1000 red blood cells), to ensure that reaction conditions were optimal for detecting subpatent levels of parasitaemia, and a known negative (blood from either a laboratory-raised hatchling or a lizard from a malaria-free site). All samples scored as negative after microscopic examination, but positive by PCR, were re-examined by an additional 6-min microscope scan as well as an additional nested PCR reaction to confirm that they were indeed positive, yet undetectable on the slide.

Results

All 59 lizards scored as positive by slide scanning were also scored positive by PCR (Fig. 1). For lizards scored as noninfected by blood smear examination, 4/97 (4%) at the high-prevalence site, and 4/62 (6%) at the low-prevalence

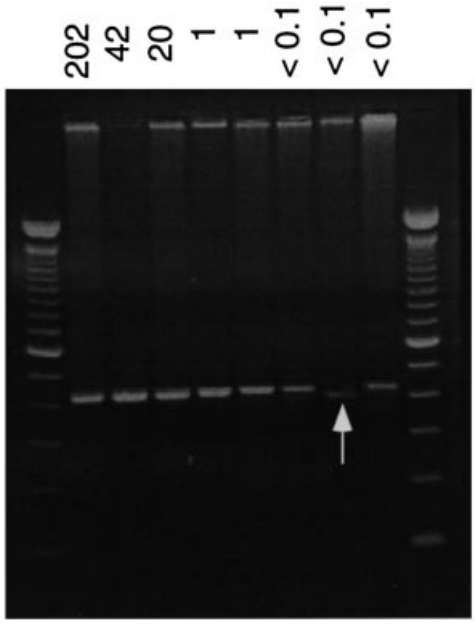

Fig. 1 Sample results for nested PCR-based detection of the malaria parasite *Plasmodium mexicanum*. The lanes include infections of various parasitaemia (the number of parasites per 1000 erythrocytes is given above each lane). The results show reliable detection of even very weak infections for the 18S rRNA gene, including those often not detected during microscopic scanning of a thin blood film (infections < 1/10 000 erythrocytes). The band indicated with an arrow is the single variant in size observed in the study. The rRNA genes of *Plasmodium* are not always homogeneous in sequence or size (Rogers *et al*. 1995).

site were revealed as infected by the nested PCR. There was no difference in number of false negatives at the two sites ($\chi 2 = 0.429$, $P = 0.48$). These data allow an estimate of the true proportion of lizards infected at the two sites. Of every 100 lizards at the high-prevalence site, 30 are scored positive by examination of blood smears, and 4% of the remaining 70 are scored positive by the PCR technique. Thus estimated prevalence is increased to 33%. Similarly, the proportion infected at the low-prevalence site is estimated as 12% using PCR rather than 6%. Therefore, 9% (3/33) of infections at the high-prevalence site were of such low parasitaemia that they were detected only by the PCR method, whereas at the low-prevalence site, fully 50% of infections (6/12) were of otherwise undetectable parasitaemia.

Of the 67 infections detected by PCR, 66 revealed a PCR product of the expected size, but one (shown in Fig. 1) produced a product ≈ 20 bp smaller.

Discussion

Extremely weak infections of *Plasmodium mexicanum* were readily detected using nested PCR of the 18S rRNA gene. The assay was more sensitive than the PCR-based method used by Feldman *et al*. (1995) in the study of *Plasmodium* in Hawai'ian birds. This is not surprising as their assay was a non-nested PCR. We also used a single pair of primers (DW1 and DW3 from Creasey *et al*. 1993) in a preliminary study of the cytochrome *b* gene of *P. mexicanum* and recorded inconsistent amplification for weak parasitaemia, and a failure to detect infections of < 1 parasite in 1000 erythrocytes.

Despite the sensitivity of the nested PCR, the technique only marginally improved the accuracy of estimated prevalence. That is, the standard 6-min scan of a single blood smear from each lizard is a reliable indication of prevalence for this parasite–host system. At the high–prevalence site, the percentage infected was increased only slightly (33% vs. 30%). The estimated prevalence was doubled at the low-prevalence site (6–12%), but it would still be rated as low compared to other sites at the Hopland study area (Schall & Marghoob 1995). The differences in prevalence observed among sites at Hopland (0–50%), and the changes in prevalence among years reported earlier (Schall & Marghoob 1995), can thus be assumed to result from actual differences in prevalence rather than differences in prevailing parasitaemia.

The proportion of false negatives after blood smear scanning in this study (4–6% of all potential hosts) is lower than that reported for *Plasmodium* infections of birds (2–24%; Feldman *et al*. 1995) and humans (9–67%; Kain *et al*. 1993; Ntoumi *et al*. 1995; Bottius *et al*. 1996; Khoo *et al*. 1996). Perhaps the reptilian immune system is less proficient at limiting malarial infections, such that few infections are reduced to subpatent densities in the vertebrate host's blood. This conclusion, however, contrasts with results from our low-prevalence site where, although false negatives were uncommon, they still made up 50% of all the rare infections. Thus, infections with very weak parasitaemia are the rule at the site where infections are rare and presumably transmission is uncommon (vectors may be rare or bite infrequently). Such situations were predicted by Ewald (1994) who proposed that rare transmission would select for parasite genotypes that sustain low parasitaemia and thus extract a minor cost from their host. Also, low transmission intensity would yield few parasite genotypes per host. This could result in reduced competition between genotypes within individual hosts to ensure transmission and thus favour slowly reproducing strains.

This speculation assumes that life-history traits (such as infection growth rate and final parasitaemia) vary adaptively in *Plasmodium*. Substantial variation in life-history traits has been observed both within and among species of malaria parasites (Bromwich & Schall 1986). Most intriguing are cases of sequestration of most parasite cells,

with very low parasitaemia occurring in the blood (e.g. Telford 1996). As only gametocytes in the blood can be transmitted by biting vectors, this presents a conundrum in the life history of such populations of *Plasmodium* (Taylor & Read 1997) which can be resolved only by a very sensitive assay for the true prevalence of subpatent infections, such as the nested PCR described here.

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