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DETECTING AVIAN MALARIA: AN IMPROVED POLYMERASE CHAIN REACTION DIAGNOSTIC

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ABSTRACT: We describe a polymerase chain reaction (PCR) assay that detects avian malarial infection across divergent host species and parasite lineages representing both *Plasmodium* spp. and *Haemoproteus* spp. The assay is based on nucleotide primers designed to amplify a 286-bp fragment of ribosomal RNA (rRNA) coding sequence within the 6-kb mitochondrial DNA malaria genome. The rRNA malarial assay outperformed other published PCR diagnostic methods for detecting avian infections. Our data demonstrate that the assay is sensitive to as few as 10^{-5} infected erythrocytes in peripheral blood. Results of avian population surveys conducted with the rRNA assay suggest that prevalences of malarial infection are higher than previously documented, and that studies based on microscopic examination of blood smears may substantially underestimate the extent of parasitism by these apicomplexans. Nonetheless, because these and other published primers miss small numbers of infections detected by other methods, including inspection of smears, no assay now available for avian malaria is universally reliable.

Avian malaria is caused by a group of apicomplexan species of *Plasmodium* and *Haemoproteus*, which infect, among other tissues, peripheral red blood cells of their vertebrate hosts. Historically, avian malaria has been detected by microscopic examination of blood smears (Greiner et al., 1975; McClure et al., 1978; Peirce, 1981; Atkinson and Van Riper, 1991). More recently, the development of molecular technology has made screening for these parasites faster and more reliable. Interest in developing a single screening assay that accurately detects diverse strains of nonhuman malaria, including subpatent infections missed by microscopic examination, has resulted in a number of polymerase chain reaction (PCR) assays (Feldman et al., 1995; Li et al., 1995; Perkins et al., 1998; Bensch et al., 2000; Jarvi et al., 2002; Richard et al., 2002) and a serological technique (Atkinson, Dusek et al., 2001).

Although serology may be more sensitive than PCR (Jarvi et al., 2002), serological methods recognize antibodies to malaria rather than detecting the parasites themselves. Therefore, using this technique to interpret positive results as current infections depends on the assumption that birds carry the disease throughout their lifetime. Isolated birds in captivity have been shown to maintain infections for at least 4.4 yr, demonstrating antibodies even when parasites are not detectable by microscopy or PCR screening (Atkinson and Van Riper, 1991; Atkinson, Dusek et al., 2001; Atkinson, Lease et al., 2001; Jarvi et al., 2002). The later recurrence of visible infection in the same birds implies that although infections may become latent in tissues other than peripheral blood, the malarial parasites are not eliminated (Atkinson, Dusek et al., 2001). However, few long-term studies are available to test this assumption in wild populations. If birds sometimes clear malarial infections, the diagnostic effectiveness of serology would be compromised because current infections, whether latent or present in peripheral blood, cannot be distinguished from historical (cleared) infections to which antibodies remain in circulation. In contrast, microscopy and PCR of DNA extracted from blood detect only active infections. Thus, serology and PCR have different, and only partially overlapping, utility.

The published PCR assays for avian malaria amplify different regions of the parasites' genome. Two assays (A and B) were designed in the nuclear-encoded 18S small subunit (SSU) of ribosomal RNA (rRNA) (Feldman et al., 1995; Li et al., 1995). Later, Bensch et al. (2000) described a PCR assay (C) based on the mitochondrially encoded cytochrome *b* gene. A fourth assay (D) was developed in our laboratory and also is based in the cytochrome *b* gene (see Richard et al., 2002). PCR assays have since been developed specifically for *Plasmodium relictum* (Jarvi et al., 2002). Perkins and Schall (2002) described additional primers used to sequence the cytochrome *b* gene in birds and reptiles. The use of these primers to screen avian blood samples for malarial infections has not been evaluated, and they are not considered here.

Richard et al. (2002) performed a comparative analysis of PCR assays A–D plus microscopic reading of blood smears for detecting avian malaria. The authors concluded that the 2 primer sets designed within the cytochrome *b* gene (C and D) were superior in detecting infections in comparison with any of the other 3 methods. Furthermore, they found that the 2 assays based on the nuclear-encoded rRNA sequences were ineffective when applied to their dataset.

Although the PCR assays developed within the cytochrome *b* gene outperformed the other assays, this gene in malarial parasites has a high AT content (approximately 73%), making it difficult to design effective primers. In addition, designing 'universal' primer assays is complicated by sequence variation in *Plasmodium* spp. and *Haemoproteus* spp. cytochrome *b* (Bensch et al., 2000; Richard et al., 2002; Ricklefs and Fallon, 2002; Waldenström et al., 2002). In this study, we describe a new PCR assay based on a highly conserved region of the mitochondrial SSU and large subunit (LSU) rRNA genes. We then compare the new assay with cytochrome *b*–based assays C and D and with visual inspection of blood smears for a sample of Neotropical passerine birds and doves. We show that the new PCR primers are conserved across a wide variety of genetically differentiated parasite lineages and outperform the previously published primers for detecting avian malaria. However, despite their increased specificity, the new primers failed to detect a small number of infections. Thus, these primers do not represent a universal assay for the presence of avian *Plasmodium* spp. and *Haemoproteus* spp. in peripheral blood.

MATERIALS AND METHODS

We designed primers within the highly conserved region of the 6-kb mitochondrial (mtDNA) genome that contains fragments of the SSU

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        10         20         30         40         50
CAGGACGTTC TTAACCCAGC TCACGCATCG CTTCTAACGG TGAACTCTCA
TTCCAATGGA ACCTTGTTCA AGTTCAAATA GATTGGTAAG GTATAGCGTT
TACTATCGAA TGAAACTATG TGTTCCACCG CTAGTGTTTG CTTCTAACAT
TCCACTTGCA TTATAACTGT ATGGACGTAA CCTCCAGGCA AAGAAAATGA
CCGGTCAAAA CGGAATCAGT TAACTATGGA TAGCTGATAC TAGCAATTTA
TCATTACTCA AGTCAGCATA GTCTATATGA AGGTTT
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FIGURE 1. Consensus sequence of 286 bp of the mitochondrial SSU and LSU rRNA genes for 5 avian *Plasmodium* s.l. and *Haemoproteus* spp. malarial parasites. The locations of the 343F and 496R RNA primers are indicated by gray shading. Variable sites are indicated by boldface type.

and LSU rRNA genes, based on 5 complete, published sequences for mammalian *Plasmodium* mtDNA (GenBank AF014115, AF014116, AF05587, AJ29878, M2900). Because of sequence divergence between mammalian and avian malarial genomes, amplification using these initial primers was inconsistent and required both a hot start and a touch down PCR protocol to amplify avian parasite DNA. To increase the specificity of our PCR assay while allowing for broad applicability across parasite lineages, we sequenced this rRNA region for 5 genetically divergent avian parasites: lineages 1, 28, 32, 43, and 54 from Ricklefs and Fallon (2002). These 5 parasite lineages were recovered from 4 distinct host families including both passerines and nonpasserines: Muscicapidae, Vireonidae, Fringillidae, and Anatidae. The lineages were selected to represent the breadth of the avian malarial phylogeny. They span up to 12% sequence divergence, equivalent to the deepest known node in the avian *Haemoproteus* spp. and *Plasmodium* spp. tree, based on cytochrome *b* sequences (Ricklefs and Fallon, 2002).

From the resulting sequences of the rRNA region for these 5 lineages, we designed primers specific to avian *Plasmodium* spp. and *Haemoproteus* spp. parasites. We amplified and sequenced 15 PCR products from a variety of infected birds based on the new primers to confirm the amplification of mitochondrial SSU and LSU rRNA genes representing avian malarial parasite DNA. We named the original flanking primers, which were based on mammalian *Plasmodium* spp. 292F (5′CGGTAGATAGGGAACAAACTGC3′) and 631R (5′GCGAGAAGGGAAGTGTGTTTC3′), and the final pair of avian malaria primers, 343F (5′GCTCACGCATCGCTTCT3′) and 496R (5′GACCGGTCATTTTCTTTG3′). Primers are numbered according to the published nucleotide positions of the 6-kb mitochondrial genome for *P. vivax* (AF05587).

PCR reactions were run in a 10-μl volume that contained the following components in their final concentration: 2.0–2.5 mM $MgCl_2$, 0.2 mM each deoxynucleoside triphosphate, 0.4 μM each primer, and 0.5 units of Qiagen Taq polymerase (Qiagen, Inc., Valencia, California). One microliter of the DNA extraction solution was used for amplification. Cycling conditions for the original flanking primer pair (292F and 631R) followed a hot-start, touch-down protocol, and were as follows: 2 min at 94 C, followed by 35 cycles with 1-min denaturation at 94 C, 1-min annealing at 52–42 C, and elongation at 72 C for 1 min and 10 sec. After the 35 cycles, a final elongation step followed at 72 C for 3 min. The final assay primer pair (343F and 496R) was run using the following protocol: 2 min at 94 C, followed by 35 cycles with 1-min denaturation at 94 C, 1-min annealing at 57 C, and elongation at 72 C for 1 min and 10 sec, with a final elongation step at 72 C for 3 min. Bands were observed on 1.0% agarose gels.

After gel purification, sequencing was carried out on an automated sequencer (ABI Prism 377, Applied Biosystems, Foster City, California) according to the manufacturer's protocol. All sequences were edited and aligned using DNASTAR® software (DNASTAR, Inc., Madison, Wisconsin).

To compare the effectiveness of PCR assays, we sampled 100 individuals of 18 species of passerine birds and doves collected in the Guanica Forest, southwestern Puerto Rico, during January 2001. This bird community is known to harbor a wide diversity of parasite lineages (data not shown); however, none of the lineage used in the design of the primers was obtained in Puerto Rico. Therefore, the sample used to test the PCR assays is independent of their design and should reflect results more generally. We caught birds using mist nets and collected 5–10 μl of blood by wing venipuncture. Smears were prepared using approximately 2–3 μl of blood. Slides were air-dried, fixed in absolute methanol, and stained with modified Giemsa stain solution (Sigma Chemical Co., St. Louis, Missouri). The remainder of the blood sample was stored in Puregene® cell lysis buffer before DNA extraction by salt precipitation according to the manufacturer's protocol (Gentra Systems, Minneapolis, Minnesota). All samples were subjected to 3 PCR assays. Two of the assays were previously described by Richard et al. (2002) and are based on the HaemF–HaemR primer pair (see also Bensch et al., 2000) and the 621F–983R primer pair. The third assay (343F–496R), referred to as the RNA assay in this study, is described above. Malarial infections were screened visually on blood smears by examining approximately 5,000–10,000 erythrocytes using a Zeiss Axioskop microscope at ×400 magnification under oil immersion.

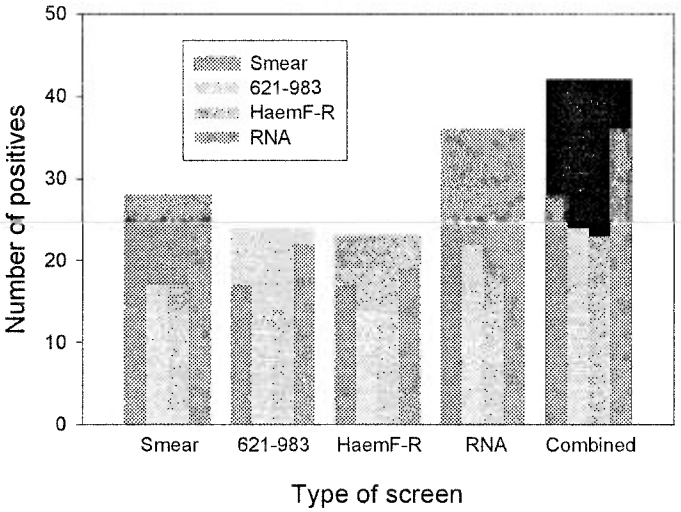

FIGURE 2. Number of positive *Plasmodium* spp. and *Haemoproteus* spp. infections in 100 birds representing 18 species sampled in the Guanica Forest, southwestern Puerto Rico, during January 2001. Infections were assayed by visual inspection of blood smears and PCR amplification using assays based on 3 pairs of avian malaria–specific primers. The wide bars indicate the number of positives for each type of screening. The narrower bars indicate the number of those positives also detected by the other 3 methods. The black bar at the right indicates the number of individuals found positive by 1, or more, of the screening methods.

To determine the lower limits of PCR detection of malarial parasites in avian blood, we conducted serial 1:9 dilutions of 3 samples of DNA extracted from infected blood. Given that our DNA samples represent a mixture of parasite and host DNA, we controlled for PCR primer and DNA template interactions by using a dilutant containing bird DNA from uninfected blood. In this way, only the parasite DNA was diluted and not that of the host. Intensities of parasite infections in the 3 samples were determined by visual inspection of blood smears (15 fields at ×400 magnifications: 10 to 25 × 10^3 erythrocytes). PCR reactions were run for each of the diluted samples, and products were run on 1% agarose gels. Bands were quantified on a Kodak Image Station 440 CF gel scanner and expressed relative to the band intensity of the undiluted sample of 1 of the infected birds (A in Fig. 3).

RESULTS

The mitochondrially encoded RNA region (Fig. 1) for the 5 genetically distinct avian parasite lineages was nearly 6 times more conservative than the cytochrome *b* gene for the same 5 lineages. The average uncorrected sequence divergence was 1.5% compared with 8.9% for cytochrome *b*. In addition, the GC content of the RNA sequences (40%) was 13% higher than that of the cytochrome *b* sequences.

The combined results from all 4 diagnostic methods revealed a total of 42 infections (Fig. 2). The 4 methods produced con-

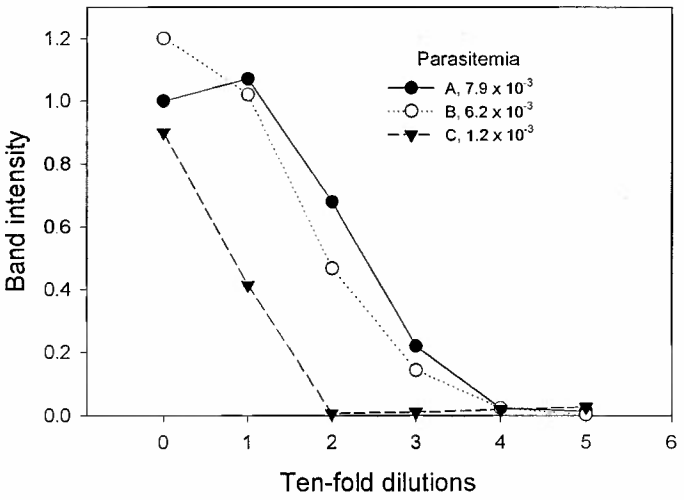

FIGURE 3. PCR product concentrations (band intensities) of avian malarial DNAs after 10-fold serial dilutions of the original DNA extractions using the RNA screening primers described in this study. Samples A and B were obtained from Puerto Rican Bullfinches (*Loxigilla portoricensis*), and sample C was obtained from a Bananaquit (*Coereba flaveola*). Parasitemias are number of infected cells per 1,000 erythrocytes obtained by scanning 15 fields at ×400 magnification. Number of scanned erythrocytes was estimated to be 9,400, 25,900, and 14,900.

sistent results for 68 samples, i.e., 10 positive and 58 negative. For the 32 individuals that were not consistently assayed by the 4 diagnostic techniques, we obtained a variety of assay outcomes regarding the presence or absence of malarial parasites. In the 7 cases where only 1 method identified a positive infection, the RNA primers identified 5 positives, and each of the 2 other primer pairs uniquely identified 1 positive. As a measure of reproducibility, we reassayed all RNA-positive infections with the RNA primers. All but 2 samples reproduced their original result. Infection in these 2 samples was not detected by any other method, and they represent hosts that rarely, but occasionally, demonstrate infection. The RNA primers failed to detect 6 infections, 4 of which were visually detected on slides, 4 with HaemF–HaemR, and 2 with 621F–983R. Inspection of slides failed to detect 14 infections, 12 of which were picked up by the RNA primers. Of the other 2, one was detected by HaemF–HaemR, and the other was detected by 621F–983R.

We performed serial 10-fold dilutions on 3 avian DNA samples, with infections quantified by examining blood smears. Variation in band intensity of the RNA PCR products corresponded roughly to the intensity of malarial infection (Fig. 3). For 2 samples with parasitemias of 6×10^{-3} and 8×10^{-3} per erythrocyte, the RNA primers amplified parasite DNA after 1,000-fold dilution, which was the equivalent of less than 1 infected cell in 100,000 (10^{-5}/erythrocyte). For a third sample with an initial parasitemia of about 1×10^{-3}, the RNA primers detected infection only in the first 10-fold dilution, equivalent to 1 parasite per 10^4 erythrocytes.

DISCUSSION

The RNA region of the mitochondrial genome of avian malarial parasites is highly conserved, making it an excellent location to design PCR primers for detecting infections. The PCR assay described in this study can detect both *Haemoproteus* spp. and *Plasmodium* spp. that differ by up to 12% sequence divergence in the cytochrome *b* gene (Ricklefs and Fallon, 2002). By design, this assay is globally applicable, and it has detected infections in both passerine and nonpasserine hosts from a variety of locations including Europe, North America, Africa, and Australia (data not shown). Despite the versatility of these new primers, a small number of visible infections and some infections detected by other avian malarial PCR primers were missed, indicating that the new RNA primers are not universal for bird parasites. Nonetheless, the new RNA assay appears to be more effective in screening for infections than examination of smears or previously published PCR assays and when used in conjunction with I, or more, of these other assays, is likely to provide a reliable assessment of infections present in circulating blood.

Because the birds that we screened in comparing the methods were wild caught, we could not assess false negatives in our dataset. False negatives are likely the result of variation in priming sites between divergent lineages and may also reflect low parasitemias. Our serial dilutions suggest that the lower limit of detection is on the order of 10^{-4}–10^{-5} parasites per erythrocyte. However, the fact that these primers miss some infections indicates that the true prevalence of avian malaria is even higher than that reported in this study.

Serological assays may indicate higher prevalence of infection than inspection of smears and PCR methods. However, the comparability of these methods depends on whether birds clear infections or whether antibody concentrations drop with time. Experiments with native Hawaiian birds, which are highly vulnerable to malarial infection, show that individuals can resist reinfection for at least 2 yr after an initial infection, and that antibodies can be detected up to 4 yr after initial infection (Atkinson, Dusek et al., 2001). Malarial parasites in some individuals kept in caged conditions and not susceptible to reinfection could be detected by PCR of DNA extracted from blood for over 2 yr, indicating the long-term persistence of active infections (Jarvi et al., 2002). However, it is unclear whether birds normally exposed to malarial parasites can clear infections, at least from peripheral blood. At this time, it is difficult to distinguish birds without infection from birds with infections that are restricted to internal organs and absent from blood. PCR assays of DNA extracted from blood are negative for both; serological assays could be positive for both. Estimates of parasite prevalence based on smear and PCR assays represent minimum levels of infection, whereas estimates based on serology may represent maximum levels of infection and probably stand as a record of all individuals ever infected.

Regardless of the ambiguity in infection rates recorded by PCR assays, visual examination of blood smears, and serology, it is clear that new diagnostic techniques continue to reveal a higher prevalence of avian malarial parasites than previously appreciated. Thus, because of the pervasive nature of avian malarial infection, the effects of these parasites on the ecology and evolution of their hosts need to be reconsidered. Intense infections that can be readily detected on blood smears may reflect a weakened state of the host's immune system or infection by a strain of malaria having particularly high virulence, rather than the absolute presence or absence of infection. In any case, understanding the true nature of avian malarial infections will

require the application of molecular diagnostics such as the mitochondrial RNA assay presented in this study.

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