

A Comparative Analysis of PCR-Based Detection Methods for Avian Malaria

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ABSTRACT: Here, 4 polymerase chain reaction (PCR) assays are compared to test for the presence of avian malaria, including both the *Plasmodium* and *Haemoproteus* genera, in 29 different species of African rainforest birds. Two of these PCR assays use primer sets that amplify fragments of the cytochrome *b* (*cyt b*) gene of *Plasmodium*; the other 2 target the 18S ribosomal subunit gene. These PCR assays were performed using genomic DNA extracted from blood and subsequently compared with the results obtained by microscopic examination of blood smears taken from the same individuals. The 2 primer sets amplifying the *cyt b* gene were found to perform more reliably than those that target the 18S rRNA gene and yielded a substantial number of positive samples that were undetected by blood smear analysis. Of all the individuals screened by PCR, 40% tested positive for avian malaria, whereas 27% tested positive by blood smear analysis. Although sequence variation in the parasites may prohibit the specific alignment of primers and the subsequent PCR amplification of some individuals, PCR, once optimized, is faster, cheaper, and more reliable than blood smear analysis for large-scale screening.

affects a wide range of other mammals, reptiles, and birds (Cook, 1971; Olsen, 1974). Avian malaria is caused by species of *Plasmodium* and *Haemoproteus*, and all of these parasites have been found in African rainforest birds (Kirkpatrick and Smith, 1988).

Typically, avian malaria has been screened for by microscopy. A positive diagnosis depends upon the visualization of parasites in Giemsa-stained blood smears (Kirkpatrick and Smith, 1988; Payne, 1988). Whereas this method typically meets with adequate success, it suffers from significant drawbacks, including the time-intensive scanning of slides and the potential for low-level infections to avoid detection. Recent advances in the detection of malaria through the use of the polymerase chain reaction (PCR) have alleviated many of the problems associated with traditional slide scanning, yielding greater sensitivity and faster results (Li et al., 1995; Tham et al., 1999).

The first published account of a PCR assay for a form of avian malaria explored the prevalence of *P. relictum* in Hawaiian honeycreepers (Fringillidae: Drepanidinae), a particularly vulnerable family of birds, and used primers that targeted the gene encoding the 18S subunit of ribosomal RNA (Feldman et al., 1995). Another set of primers was developed by Li et al. (1995) who identified several conserved regions

Malaria, one of the most devastating diseases to affect humans, also

TABLE I. Results of 3 PCR assays and microscope examinations.

| Primer sets | Samples tested | Positive by microscopy | Negative by microscopy | Microscopy positive, PCR negative | Microscopy negative, PCR positive |
|------------------------------------|----------------|------------------------|------------------------|-----------------------------------|-----------------------------------|
| 566, 570, and 841, 844* (18S rRNA) | 53 | 19 | 37 | 5 | 4 |
| HAEMF, HAEMR† (cyt <i>b</i>) | 189 | 51 | 138 | 4 | 20 |
| 621, 983‡ (cyt <i>b</i>) | 189 | 51 | 138 | 7 | 9 |

* Li et al. (1995).

† Bensch et al. (2000).

‡ This study.

in the small subunit RNA of *Plasmodium* sp. Whereas their nested PCR tests focused exclusively on human strains of the parasite, it was assumed that their universal primers would successfully detect a range of *Plasmodium* species, including those that utilize avian hosts. In fact, Perkins et al. (1998) used the same primers to detect malaria infections in the western fence lizard *Sceloporus occidentalis*. More recently, Bensch et al. (2000) presented results from primers based on conserved regions of the cytochrome *b* (cyt *b*). Although their work focused primarily on building a phylogeny of *Plasmodium* and *Haemoproteus* strains, they also included a comparison between their PCR assay and results of blood smear analyses. The present work describes yet another primer set that also targets a region of the cyt *b* gene (S. Fallon and R. Ricklefs, unpubl. data).

With these available primers sets, an effort was made to compare their effectiveness for use on blood samples of West African rainforest birds. Specifically, the goals were to compare the 4 PCR assays to determine which was most effective in detecting West African malaria parasites and to compare the results of the PCR assays to those found by slide scanning. The most successful PCR tests were then employed to determine the incidence of avian malaria in 29 species of African rainforest birds. Each of the 4 PCR assays was performed using unique reaction conditions optimized for each primer set on the basis of the original protocols.

The blood samples used in this study were collected opportunistically as a part of an ongoing study of avian evolution in Central Africa (Smith et al., 1997, 2000). Blood samples for molecular assays were collected in lysis buffer (10 mM Tris-HCl, pH 8.0, 100 mM EDTA, 2% SDS). To obtain total DNA, the blood was either extracted following a DNeasy kit protocol (Qiagen, Valencia, California) or with phenol-chloroform followed by ethanol precipitation (Kocher et al., 1989). Blood smears were made on site, air dried, fixed in methanol, and then placed in a 3% Giemsa solution for staining. They were subsequently examined using an Olympus BH compound microscope at $\times 200$, $\times 400$, and $\times 1,000$ resolutions for 20–50 min. At least 10,000 erythrocytes were examined per slide at $\times 1,000$ magnification. The presence of malarial parasites (both *Plasmodium* spp. and *Haemoproteus* spp.) was then recorded.

The first primer set used in the comparative analysis, developed by Feldman et al. (1995), targets the 18S rRNA gene of both the host bird and parasite. Because the parasite 18S rRNA sequence includes a 200 base pair (bp) insertion, a positive result is evident by observing 1 band at 380 bp (avian) and 1 at 580 bp (malarial). A negative test would yield solely the 380-bp band. The primers for this assay were designated as: (90) 5'-GCATGGCCGTTTTAGTTCGTGAAT-3' and (89) 5'-TATCTTCAATCGGTAGGAGCGACG-3' by Feldman et al. (1995). DNA was amplified in a 25- μ l reaction mixture with 2.5 μ l 10 \times PCR buffer II (100 mM Tris-HCl, pH 8.3, 500 mM KCl), 1.5 mM MgCl₂, 2.0 mM each dNTP, 1.0 mM of each primer, and 0.625 units of Amplitaq (Applied Biosystems, Inc., Foster City, California) per sample. The reaction mixture was run through 30 cycles of 94 C for 40 sec, 48 C for 2 min, and 72 C for 45 sec, followed by extension at 72 C for 10 min.

The second, a nested assay, amplifies the 18S rRNA gene with 2 sets of primers described by Li et al. (1995). The first set is: (570) 5'-CGACTTCTCCTTCTTTAAAAGATACG-3' and (566) 5'-GGA-TAACTACGGAAAAGCTGTAGC-3'. A 25- μ l reaction mixture with 2.5 μ l 10 \times PCR buffer II (described earlier in the article), 3.0 mM MgCl₂, 400 μ M each dNTP, 0.7 μ M of each primer, and 0.625 units

Amplitaq per sample was heated to 95 C for 5 min and then run through 35 cycles at 95 C for 1 min, 48 C for 1 min, and 72 C for 2 min. An aliquot of this amplified product (1 μ l) was then used to seed the second round, which uses the primers (841) 5'-GAACGAGATCTTAACCTGC-3' and (844) 5'-TATTGATAAAGATTACCA-3' and the same reaction conditions, except that the annealing temperature was raised to 50 C. The presence of an approximately 400 bp product was scored as a positive detection of avian malaria.

The third set of primers examined was specific for a segment of the cyt *b* gene. The primers designated HAEMF (5'-ATGGTGCTTTCGATATATGCATG-3') and HAEMR2 (5'-GCATTATCTGGATGTGATAATGGT-3') by Bensch et al. (2000) were used in a 25- μ l reaction mixture consisting of 2.5 μ l 10 \times PCR buffer II (described earlier in the article), 1.5 mM MgCl₂, 400 μ M each dNTP, 0.6 μ M of each primer, and 0.625 units Amplitaq per sample. The reaction mixtures were heated for 3 min at 94 C and subsequently run through 35 cycles of 94 C for 30 sec, 55 C for 30 sec, and 72 C for 45 sec. The samples were then incubated at 72 C for 10 min. The presence of a 478-bp fragment indicated a positive result.

The fourth and final set of primers are described for the first time here and are designated (621) 5'-AAAAATACCCTTCTCAATCCAAATCT-3' and (983) 5'-CATCCAATCCATAATAAAGCAT-3' (S. Fallon and R. Ricklefs, unpubl. data). These primers were designed from *Plasmodium* cyt *b* sequences for parasites of mammals deposited in GenBank. They were used in a 25- μ l reaction mixture consisting of 2.5 μ l PCR buffer II (described above), 3.0 mM MgCl₂, 400 μ M dNTP, 0.2 μ M of each primer, and 0.625 units Amplitaq per sample. The reaction mixtures were heated for 90 sec at 94 C, 40 sec at 50 C, and 70 sec at 72 C. They were subsequently run through 35 cycles of 94 C for 30 sec, 50 C for 40 sec, and 72 C for 70 sec. One final cycle was used consisting of 94 C for 30 sec, 50 C for 40 sec, and 72 C for 3 min. The presence of a 341-bp fragment was interpreted as a positive result.

Brain-derived neurotrophic factor (BDNF) primers were used as a positive control to test the success of DNA extractions and are designated ChickBDNF5' (ATGACCATCCTTTTCTTACTATG) and ChickBDNF3' (TCTTCCCCTTTTAAATGGTTAATGTAC). Each 25 μ l reaction mixture contained 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 4.0 mM MgCl₂, 0.001% gelatin, 200 μ M of each dNTP, each primer at 0.4 μ M, and 1 unit Amplitaq. Reaction conditions were: an initial 5 min denaturation at 94 C; 35 cycles of 30 sec denaturation at 94 C, 30 sec annealing at 55 C, and 30 sec extension at 72 C, followed by 7 min extension at 72 C.

For the detection of malaria, in each experiment positive and negative controls were included. Positive controls consisted of a dilute solution of purified *Plasmodium falciparum* genomic DNA and known infected samples (based on results of blood smear analyses) of both *Plasmodium* and *Haemoproteus*. The samples used as positive controls included a range of intensities from several different avian species. Negative controls were DNA samples of birds shown to be clear of infection by microscopy and repeated PCR screening. These controls were used for all primer sets. In all cases, the amplified PCR products were visualized on 2% agarose gels using ethidium bromide under UV light.

To obtain longer DNA fragments, we used primer HAEMF and primer 983 to amplify a \sim 700 bp fragment of the cyt *b* gene using the reaction conditions described earlier in the article for HAEMF and HAEMR2 and Bensch et al. (2000). PCR products were purified using a Qiagen® kit following manufacturer's instructions (Qiagen). Bidirec-

TABLE II. Individuals infected with avian malaria by species.

| Family and species | Infected/Total |
|-----------------------------------|----------------|
| Alcedinidae | |
| <i>Alcedo quadribrachys</i> | 0/1 |
| <i>Alecedo leucogaster</i> | 1/3 |
| <i>Ceyx lecontei</i> | 1/1 |
| Capitonidae | |
| <i>Pogoniulus subsulphareus</i> | 0/1 |
| Corvidae | |
| <i>Picathartes oreas</i> | 0/1 |
| Estrildidae | |
| <i>Spermophaga haematina</i> | 0/1 |
| Motacillidae | |
| <i>Motacilla clara</i> | 1/1 |
| Muscicapidae | |
| <i>Platysteira concreta</i> | 1/2 |
| <i>Terpsiphone rufiventer</i> | 2/2 |
| <i>Trochocercus nigromitratus</i> | 0/1 |
| Nectariniidae | |
| <i>Nectarinia olivacea</i> | 41/68 |
| Picidae | |
| <i>Verreauxia africana</i> | 0/1 |
| Pycnonotidae | |
| <i>Andropadus latirostris</i> | 10/59 |
| <i>Andropadus virens</i> | 1/7 |
| <i>Bleda canicapilla</i> | 3/4 |
| <i>Bleda eximia</i> | 1/6 |
| <i>Bleda syndactyla</i> | 0/2 |
| <i>Criniger chloronotus</i> | 1/4 |
| <i>Phyllastrephus icterinus</i> | 1/1 |
| <i>Phyllastrephus xaveri</i> | 2/2 |
| Sylviidae | |
| <i>Hylia prasina</i> | 0/2 |
| Timaliidae | |
| <i>Illadopsis cleaveri</i> | 0/1 |
| <i>Illadopsis rufipennis</i> | 0/3 |
| Turdidae | |
| <i>Alethe diademata</i> | 1/1 |
| <i>Alethe poliocephala</i> | 5/6 |
| <i>Neocossyphus poensis</i> | 2/3 |
| <i>Sheppardia cyornithopsis</i> | 0/2 |
| <i>Stiphornis erythrothorax</i> | 0/2 |
| <i>Stizorhina fraseri</i> | 1/1 |
| Total of 29 bird species | 75/189 (40%) |

tional sequencing of the ~700 bp PCR fragments was performed using the primers HAEMF and primer 983 in an ABI Prism 377 automated sequencer (Applied Biosystems). We used double-stranded cycle sequencing with dye-terminator fluorescent labeling and electrophoresed sequenced products through a 5% Long Ranger gel (Sehgal et al., 2001).

Primers 89 and 90 (Feldman et al., 1995) did not function effectively on these samples; multiple bands were observed when using the published protocol and several other reaction conditions. One possible explanation is that the 18S rRNA gene sequences of the malaria parasites infecting African birds may be significantly divergent from those of the *Plasmodia* species that infect Hawaiian honeycreepers. This primer set was not used further in the present study.

Table I illustrates the efficacy of the remaining 3 PCR tests for detecting avian malaria. It compares the tests with blood smear analysis by microscopy and details the number of false negatives and false positives detected by each method. The primer sets of the nested PCR protocol (566, 570, and 841, 844 [Li et al., 1995]) were only used for an initial screening of 53 samples. The nested assay, although capable of detecting purified *P. falciparum*, often did not detect known positive infections of both *Haemoproteus* and *Plasmodium* and did not demonstrate consistent repeatability in those samples that scored positive. In addition, this protocol suffers from the requirement of additional time and resources caused by the 2 rounds of PCR amplification.

Primer sets HAEMF-HAEMR (Bensch et al., 2000) and 621 and 983 (described here) proved to be far more effective at detecting avian malaria within the samples. These PCR assays were therefore used to screen 136 additional blood samples (see Table I). The assay using primers 621 and 983 detected 9 false negatives and the HAEMF and HAEMR primers 20. Four samples that were scored as positive by slide scanning did not prove to be positive with the HAEMF and HAEMR primers, as compared with 7 for the 621 and 983 primers. Serial dilution experiments suggested that there was no detectable difference between the 2 primer sets' abilities to detect a minimum threshold of avian malaria (data not shown).

The occurrence of false negatives by PCR could be caused by an insufficient concentration of malarial DNA in the sample or even a failure of the extraction itself. All samples classified as false negatives were tested at least twice (with positive controls present) to ensure that the negative results were not caused by PCR failure. When available, multiple extractions of the same sample were tested to improve the likelihood of detection. To ascertain that the extractions were indeed successful, the contentious samples were tested by PCR for a nuclear avian gene (the BDNF) to ensure that DNA was present, and in all cases amplification did occur.

With the results obtained by PCR, the various bird species infected with avian malaria were identified, and these results are shown in Table II. In all, 75 of 189 (40%) individuals tested positive for avian malaria using the 2 methods that amplify fragments from the *cyt b* gene. In contrast, by microscopy, 51 individuals (27%) tested positive. Of the 29 species tested, 17 species (59%) harbored the parasite, using the PCR methods. By microscopy, 11 species (38%), tested positive.

Given the large number of avian species included in this survey, it was hypothesized that significant sequence variation may exist within the species of *Plasmodium* and *Haemoproteus* we observed. In order to test this hypothesis and to confirm that our positive samples were not lab contamination by *P. falciparum*, 10 positive samples were selected for sequencing. DNA fragments were obtained by amplification of a longer ~700 bp fragment of the *cyt b* gene using HAEMF and primer 983. Some species of *Plasmodium* showed overall sequence variation of >8%, including sequence in the primer binding regions in this DNA fragment (data not shown). In the light of these findings, it is not surprising that not all the primers tested were entirely effective across all strains.

Whereas the PCR assays used here have not been infallible, they have proven to be effective in detecting malarial infections in the avian populations studied here. It must be noted, however, that these assays cannot distinguish between species in the 2 genera (*Haemoproteus* and *Plasmodium*) or species of the avian malaria parasites. Further efforts could develop species-specific primers.

The results of the present study suggest that the 2 primer sets that amplify the *cyt b* gene detect avian malaria in these samples at a level superior to those achieved from blood smear analysis. However, it must be made clear that any particular pair of primers located in an evolving gene region may or may not be transferable from one species of host to another. For example the primers designed by Feldman et al. (1995) worked well for *Plasmodium* spp. in Hawaiian honeycreepers, but were not reliable in detecting the African parasites in this study. Similarly, the primers 621 and 983 described here failed to accurately detect many infected birds from Puerto Rico (S. Fallon and R. Ricklefs, unpubl. data). Care must also be taken to ensure that primers are parasite specific. One primer set that amplifies the 18S rRNA gene was used not only to detect *Plasmodium* in lizards but also nonspecifically amplified host DNA; therefore it was deemed inappropriate for screening of parasites (Perkins and Martin, 1999). Once a PCR assay has been established for a particular parasite lineage, however, it allows the rapid

screening of a large numbers of individuals in a population and, for this reason, PCR assays are preferable to blood smear analysis for detecting avian malaria because of the increased efficiency and accuracy associated with them.

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