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# NESTED CYTOCHROME *B* POLYMERASE CHAIN REACTION DIAGNOSTICS DETECT SPOROZOITES OF HEMOSPORIDIAN PARASITES IN PERIPHERAL BLOOD OF NATURALLY INFECTED BIRDS

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ABSTRACT: Some discrepancies between microscopy and PCR-based methods have been recently recorded in the diagnosis of *Leucocytozoon* spp. infection in naturally infected birds. To clarify this issue, blood samples from 109 yellow-whiskered greenbuls *Andropadus latirostris* were investigated using both the microscopic examination of blood films and a nested mitochondrial cytochrome *b* PCR. The overall prevalence of *Leucocytozoon* spp. infection was 4% after the standard microscopic examination and 17% using the PCR diagnostics. Samples from 9 randomly chosen birds that were microscopy negative, but PCR positive, were then examined microscopically by screening 2 entire blood films from each individual bird. Sporozoites of *Leucocytozoon* spp. were observed in 4 birds, and 1 gametocyte of the parasite was seen in each of 2 birds. We conclude that sensitive PCR-based diagnostics are able to detect extremely light parasitemias of circulating sporozoites and gametocytes of hemosporidian parasites. Because of the PCR detection of sporozoites of unknown fate in the peripheral circulation, conclusions regarding the distribution of hemosporidian lineages in wildlife should be made with caution. To be accepted as the lineages of successfully developing species of hemosporidians, such PCR-based information should be supported with the detection of blood stages of the parasites. The present study emphasizes the crucial need for a synthesis of information provided by the tools of traditional parasitology and molecular biology, particularly in field studies of blood parasites.

A recent comparative study (Valkiūnas, Iezhova, Križanauskienė et al., 2008) showed that both microscopic examination and polymerase chain reaction (PCR)–based diagnostics detect similar levels of prevalence *Haemoproteus, Plasmodium,* and *Leucocytozoon* species in naturally infected birds. In other words, both these methods reveal the same trends of prevalence. This information is important in investigating patterns of the distribution of blood parasites in wildlife. The authors recommended the continued use of the optical microscopy in research of hemosporidian parasites in parallel to the now widely employed PCR-based diagnostic tools.

Interestingly, both these tools showed the same prevalence for *Leucocytozoon* spp. in the majority of tested bird species, but 1 result remained equivocal from that study. Mainly, the overall prevalence of *Leucocytozoon* spp. infection (n = 472) was slightly greater in the PCR-based diagnostics (30%) than microscopic examination (25%); the difference is insignificant. However, the prevalence of *Leucocytozoon* spp. infection in the olive sunbird *Cyanomitra olivacea* (n = 193) was significantly greater using PCR-based diagnostics (48%) than microscopy (37%; Yates' corrected chi-square value is 4.25, $P < 0.05$). There was no convincing explanation for this discrepancy. Because good quality blood films were examined by skilled investigators, but gametocytes of leucocytozoids were not recorded, the following explanations for this finding are suggested. First, the intensity of *Leucocytozoon* spp. parasitemia is extremely light, so gametocytes are difficult to find using standard microscopic examination protocols. Second, it is difficult to rule out a possibility that sensitive PCR-based tools amplify the DNA of sporozoites, which are not numerous in the circulation, so usually difficult to detect using microscopy (Valkiūnas, 2005). The aim of the present study was to test these hypotheses. We speculated that longtime microscopy of blood samples, which are *Leucocytozoon* spp. positive in the PCR screening, might be helpful to clarify this issue.

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## MATERIAL AND METHODS

### Study sites and collection of blood samples

As part of a comprehensive study on the effects of deforestation on the prevalence of blood pathogens in African rainforest birds, blood samples were collected from 109 yellow-whiskered greenbuls *Andropadus latirostris* in Ghana in July 2007 (Table I). This bird species is widespread in African rainforests and widely parasitized with *Leucocytozoon* spp. (Valkiūnas et al., 2005). All birds tested were adult year-round residents (Borrow and Demey, 2005). Study sites included Agumatsa (7°1.758′N, 0°33.490′E, 269 m above sea level), Abrafo (5°21.171′N, 1°23.406′E, 170 m) and Nkwanta (5°16.912′N, 2°38.495′E, 85 m). Birds were caught with mist nets between daybreak (0600) and dusk (1700). They were ringed, bled, and released. None of them was recaptured.

The blood was taken by puncturing the brachial vein. A blood drop of approximately 2 μl was used to prepare each blood film. Two blood films were prepared from each bird; they were air-dried within 5–10 sec after their preparation. In humid environments, we used a battery-operated fan to aid in the drying of the blood films. Slides were fixed in absolute methanol in the field and then stained with Giemsa in the laboratory, as described by Valkiūnas, Iezhova, Križanauskienė et al. (2008). Approximately 50 μl of whole blood was drawn from each bird for subsequent molecular analysis. The samples were fixed in lysis buffer (Sehgal et al., 2001); they were held at ambient temperature in the field and later at −20 C in the laboratory.

### Examination of blood films and parasite morphology

An Olympus BX61 light microscope equipped with Olympus DP70 digital camera and imaging software AnalySIS FIVE was used to examine slides, prepare illustrations, and take measurements. Good quality blood films, without any features of lysis of cells and well-stained blood cells and parasites, were used for microscopic examination. The following 2 microscopy protocols were applied. First, 1 blood film from each bird was examined using the standard microscopy protocol, as described by Valkiūnas, Iezhova, Križanauskienė et al. (2008). The films were examined for 10–15 min at low magnification (×400), and then at least 100 fields were studied at high magnification (×1,000). The approximate number of screened red blood cells was $5 \times 10^5$ in each blood film; the microscopy took approximately 20–25 min. Intensity of infection was estimated as a percentage by counting the number of parasites per 10,000 erythrocytes examined, as recommended by Godfrey et al. (1987). Second, 9 birds that were *Leucocytozoon* spp. positive in the PCR-based diagnostics, but negative in the standard microscopic examination, were chosen randomly. Two blood films from these birds were examined microscopically by screening the entire films at high magnification; the microscopy of each

TABLE I. Outcome of standard microscopic examination and PCR screening for *Leucocytozoon* spp. in blood samples from yellow-whiskered greenbuls *A. latirostris* collected from Ghana, July 2007.

| Study site | No. tested | No. positive* | |
|---|---|---|---|
| | | Microscopic examination | RCR screening |
| Agumatsa | 45 | 1 (2.2) | 8 (17.8) |
| Abrafo | 24 | 0 | 0 |
| Nkwanta | 40 | 3 (7.5) | 11 (27.5) |
| Total | 109 | 4 (3.7) | 19 (17.4) |

* Number in parentheses is percentage of birds positive.

blood films took approximately 8–10 hr (hereafter termed long-time microscopy).

### Extraction of DNA, PCR, sequencing, and statistical analysis

DNA was extracted from whole blood following a DNeasy kit protocol (Qiagen, Valencia, California). Success of each DNA extraction was verified with primers that amplify the brain-derived neurotrophic factor (Sehgal and Lovette, 2003).

For *Leucocytozoon* spp. detection, we used a nested PCR method that amplifies a fragment of the cytochrome *b* gene (cyt *b*) of the mtDNA with the following primers: HaemNF1/HaemNR3–HaemFL/HaemR2L following Hellgren et al. (2004). Reactions were carried out in a 25 µl reaction mixture containing 50 ng of genomic DNA, 0.4 mM of each dNTP, 0.4 mM of each primer, 2.5 ml of CL buffer, and 0.5 units *Taq* (Qiagen). The cycling profile conditions were initial denaturation at 94 C for 3 min, followed by 20 cycles of 94 C denature for 30 sec, 50 C annealing for 30 sec, and 72 C extension for 45 sec, and then a final extension at 72 C for 10 min. We used 2 µl of the first PCR reaction as the template for the second PCR. The reaction conditions using the second primer set were identical to the first round and used the same cycling profile, but performed over 35 cycles instead of 20 cycles. Positive and negative controls were used, i.e., positive controls were from birds with known *Leucocytozoon* spp. infections evident from microscopy results, and the negative controls used purified water in place of DNA template. The PCR products were run out on a 2% agarose gel using 1 × TBE and visualized by an ethidium bromide stain under ultraviolet light. In some cases we obtained 2 different sized PCR fragments, because of the first set of primers, as described by Szöllsi et al. (2008). Thus, we used well-separating agarose gels, several positive controls, and molecular standards to facilitate separation of fragments. PCR products were purified using ExoSap (following manufacturer's instructions; USB Corporation, Cleveland, Ohio), and we identified lineages by sequencing the fragments (BigDye, version 1.1 sequencing kit) on an ABI PRISM™ 3100 automated sequencer (Applied Biosystems, Foster City, California).

Samples with sporozoites were tested by a PCR method for the possible presence of simultaneous infection of *Plasmodium* spp. and *Haemoproteus* spp. We amplified a fragment of the cyt *b* gene of the mtDNA with the following primers: L15183: 5′-GTG CAA CYG TTA TTA CTA ATT TAT A-3′ and H15730: 5′-CAT CCA ATC CAT AAT AAA GCA T-3′ (Fallon et al., 2003). The cycling profile consisted of an initial denaturation at 94 C for 3 min, followed by 35 cycles of 94 C for 50 sec, 53 C annealing for 50 sec, and 72 C extension for 60 sec, and then a final extension at 72 C for 5 min (for other details see Szymanski and Lovette, 2005).

Prevalences were compared by Yates' corrected chi-square test. A *P*-value of 0.05 or less was considered significant. The representative blood slides with sporozoites were deposited in the Institute of Ecology, Vilnius University, Vilnius, Lithuania (accession nos. 41726, 42361 NS). Accession numbers of the recorded *Leucocytozoon* spp. lineages were deposited in GenBank (DQ847239, FJ839445–FJ839451).

## RESULTS

The results of testing of the same samples by the standard microscopic examination (the first detection method) and the PCR screening are given in Table I. *Leucocytozoon* spp. were not recorded in Abrafo by either method. In Agumatsa, Nkwanta, and the overall sample including Abrafo, the PCR diagnostics detected a significantly greater number of *Leucocytozoon* spp. infections than the standard microscopic examination ($P < 0.05$, $P < 0.05$, and $P < 0.01$, respectively). Species of the parasites were not identified because of light intensity of parasitemia (<0.001%) in all microscopy-positive samples.

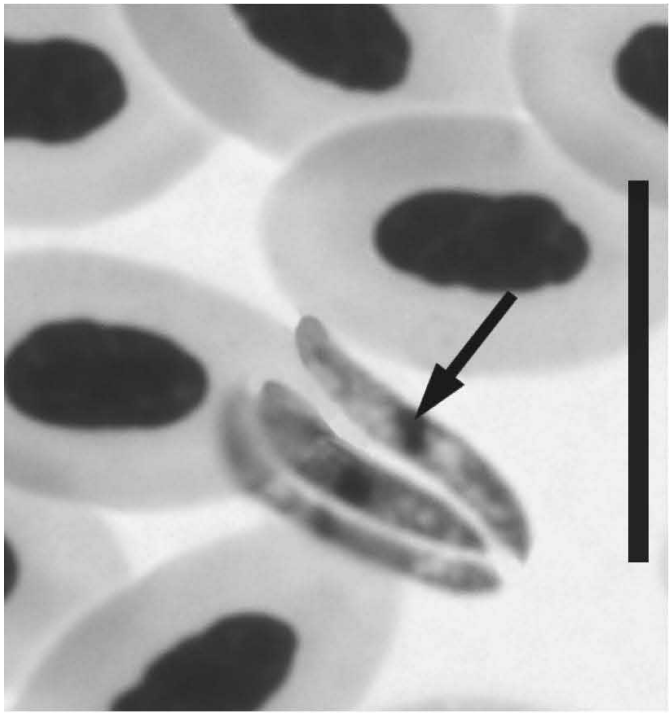

FIGURE 1. Three sporozoites of *Leucocytozoon* sp. from the peripheral blood of naturally infected yellow-whiskered greenbuls *A. latirostris*. Arrow: parasite nucleus. Giemsa-stained thin blood film. Bar = 10 µm.

During the long-time microscopy of the samples, normal-appearing *Leucocytozoon* spp. sporozoites were observed in 4 of 9 (44%) birds (Fig. 1). Up to 3 sporozoites were seen in positive blood films, both from Agumatsa and Nkwanta. It is possible that we did not detect sporozoites in blood films from other PCR-positive birds because of the very light infection. Nondeformed sporozoites (n = 6) vary between 6.0 and 9.6 (in average 8.1) µm in length and 0.9–1.4 (in average 1.2) in width; each possess a prominent centrally located nucleus (Fig. 1).

In 3 samples with sporozoites, species of *Haemoproteus* and *Plasmodium* were not detected by either the PCR method or the long-time microscopic examination, so we thus ruled out the possibility of simultaneous infections with the pigment-forming hemosporidians in these samples. In other words, the observed sporozoites certainly belong to *Leucocytozoon* spp. In 1 sample with sporozoites, *Haemoproteus vacuolatus* (cyt *b* lineage hANLA1) was detected by both the PCR method and microscopic examination (for this parasite identification see Valkiūnas, Iezhova, Loiseau et al., 2008). It is unlikely that the observed sporozoites belong to *H. vacuolatus* because gametocytes of this parasites were numerous (intensity was 0.1%) and their DNA was amplified, but gametocytes of *Leucocytozoon* sp. were not seen, so

the record of *Leucocytozoon* sp. lineage in this sample is likely due to amplification of the sporozoites' DNA.

Gametocytes of *Leucocytozoon* spp. were not seen in birds harboring sporozoites in the peripheral blood. Only 1 gametocyte of *Leucocytozoon* sp. was observed in 2 of 9 (22%) birds during the long-time microscopy. This emphasizes that the standard microscopic examination of blood films might not detect extremely light parasitemia of *Leucocytozoon* spp. gametocytes.

## DISCUSSION

There has been remarkable increase of interest of researchers in avian malaria and related hemosporidian parasites since the development of PCR-based methods for parasite detection and their species identification. New data are revealing new multiple host-parasite systems at the level of their genetic lineages (see the review by Bensch et al., 2009). It is usually deemed that PCR amplifies DNA of the developing blood stages of hemosporidians, that is, gametocytes, or erythrocytic meronts, or both; records of these blood stages clearly show that the parasites complete their life cycles in vertebrate hosts (Valkiūnas, 2005). The present study indicates that this is not always the case. DNA of sporozoites, which are injected in the blood stream of vertebrate hosts by dipteran vectors and persist in birds for some time, also can be amplified. Actually, this finding is not surprising because sporozoites of some *Leucocytozoon* species are found in the blood of experimentally infected birds for several days; live, normal-appearing sporozoites has been observed in infected birds as long as 11 days postinfection (Khan et al., 1969). The persistence of the viable sporozoites for days, or even possibly weeks, in birds helps explain the markedly asynchronous exoerythrocytic merogony of some *Leucocytozoon* spp. (Valkiūnas, 2005). The size and morphology of sporozoites recorded during present study (Fig. 1) was similar to the sporozoites of *Leucocytozoon sakharoffi, Leucocytozoon fringillinarum,* and *Leucocytozoon dubreuili,* the widespread blood parasites of passeriform birds (Fallis and Bennett, 1961; Khan and Fallis, 1970; Baker, 1971).

It is worth noting that Schultz and Whittington (2005) observed and illustrated *Plasmodium* spp. sporozoites in blood films of naturally infected South African birds. Incidentally, sporozoites of *Haemoproteus* spp. also were seen in the peripheral blood of naturally infected passeriform birds on the Curonian Spit in the Baltic Sea (G. Valkiūnas, unpubl. obs.). Thus, it is plausible to suggest that PCR-based methods can detect circulating sporozoites not only of *Leucocytozoon* spp., but also *Haemoproteus* spp. and *Plasmodium* spp.; this warrants further investigation.

It is unclear whether all sporozoites that are injected by vectors result in host infection; their development may be interrupted in resistant hosts (Valkiūnas, 2005). Because some genetic lineages of hemosporidians, which are detected from whole blood, might originate from the DNA of circulating sporozoites, the reports of such lineages do not necessarily mean that the parasites complete their development in avian hosts. Observation of hemosporidian gametocytes or erythrocytic meronts is needed to support this assertion. It is difficult to rule out the possibility that some reports of genetic lineages in avian hosts might be cases of amplification of DNA from sporozoites of unclear fate.

Because of its high sensitivity, PCR may detect a very small number of sporozoites in the peripheral circulation of small passeriform birds. Standard microscopy protocols are insufficiently sensitive to detect such light parasitemias, of both sporozoites and gametocytes; this could explain some discrepancies between levels of infection prevalence recorded using these 2 methods in parallel (Table I). It is probable that differences in *Leucocytozoon* spp. prevalence in the olive sunbird, as detected by PCR and microscopy (Valkiūnas, Iezhova, Križanauskienė et al., 2008), also might be due to the amplification of DNA from sporozoites and few gametocytes, which were undetectable during the standard microscopic examination of blood films. It is important to note that such discrepancies in *Leucocytozoon* spp. prevalence have not been reported in all bird species, and they were not recorded in species of *Haemoproteus* and *Plasmodium* (see Valkiūnas, Iezhova, Križanauskienė et al., 2008). Thus far, the discrepancies have been documented in African birds (olive sunbird and yellow-whiskered greenbul), implying a relatively high frequency of *Leucocytozoon* spp. sporozoite occurrence in the peripheral circulation of small passeriform birds in countries of warm climates with permanent transmission of hemosporidians.

An increase in the volume of whole blood used for total DNA isolation should lead to an increased probability of detecting light infections by PCR. Thus an increased extraction volume of blood would also increase the likelihood of detecting light infections, including sporozoites. This should be taken in consideration in studies comparing prevalence data of hemosporidians and other blood parasites between different sites, hosts, and studies. In other words, standardization of the whole blood volume for ecological PCR-based studies is recommended. This is currently not the case in field research of blood parasites.

In summary, the present study shows that detection of lineages of hemosporidians in vertebrate hosts should be carefully considered in field studies of blood parasites. To be accepted as the lineages of successfully developing species of hemosporidians, such PCR-based information should be supported with the detection of developing blood stages of the parasites. Conclusions regarding the distribution of hemosporidians in wildlife, based solely on the PCR-based screening of blood samples, should be made with caution. It also should be remembered that the currently used PCR-based protocols do not always read simultaneous infections of hemosporidians, which are widespread in wildlife (Valkiūnas et al., 2006). A combination of microscopic and PCR-based methods is recommended for a better understanding of the ecology of hemosporidian parasites and the epizootiology of their diseases. As molecular approaches are revealing vastly more parasite diversity than previously anticipated, the need for the synthesis of information provided by tools of traditional parasitology and molecular biology is becoming all the more crucial. We thus emphasize the value of both PCR and microscopy in studies on the distribution and ecology of avian hemosporidian parasites in wildlife.

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