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TECHNICAL NOTE

Fecal samples fail in PCR-based diagnosis of malaria parasite infection in birds

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Abstract Malaria parasites are common in wild vertebrates on all warm continents and have recently been isolated from wild apes by non-invasive fecal sampling. Here, we examined the utility of fecal samples for malaria parasite detection in wild birds. We collected both blood and fecal samples from 56 birds sampled in the field, extracted DNA from all samples using various methods, and screened all samples using sensitive PCR-based methods. We found 35 birds to be positive for malaria parasite infection (genera *Plasmodium* and *Parahaemoproteus*) using blood samples while no fecal samples revealed a positive infection. These results suggest that malaria parasites cannot be efficiently detected from fecal samples of birds and that blood sampling is still necessary for the study of the malaria parasites of wild bird populations.

Keywords Avian malaria parasites · Fecal diagnostics · Haemosporidians

The malaria parasites are an extremely successful and diverse group of blood parasites that infect mammals, squamate reptiles, crocodiles, turtles, and birds worldwide (Garnham 1966; Martinsen et al. 2008). Historically, these parasites have been studied by microscopy of blood films prepared from live vertebrate hosts. For elusive, rare, and sensitive vertebrate species, many of which are difficult to capture and sample in the field and for which sampling logistics including permitting prove difficult, parasite sampling has been limited. Recently, standard PCR-based methods have been used to detect parasite infection from non-traditional samples including archived blood and other tissue samples including those from extinct species or historical populations. The combination of PCR-based methods and non-invasive sampling techniques has allowed for parasite sampling from the great apes, a group under strict protection and for which we have limited information on their malaria parasite fauna. Using fecal samples collected in the field, a number of research groups have successfully genotyped malaria parasites of the genus *Plasmodium* from chimpanzees (*Pan troglodytes*), western gorillas (*Gorilla gorilla*), eastern gorillas (*Gorilla beringei*), and bonobos (*Pan paniscus*) (Kaiser et al. 2010; Liu et al. 2010; Prugnolle et al. 2011). Screening for malaria parasite infection from feces has also been successfully carried out in humans (Jirku et al. 2012). As birds are notoriously difficult to sample in the field, and in some cases blood sampling is not permitted or feasible, we evaluated for the first time the utility of fecal-based diagnostics for malaria parasite sampling in wild birds.

From a total of 56 wild birds caught on the grounds of the National Zoological Park in Washington, D.C. in 2010, we collected both fecal and blood samples and submerged them in lysis buffer (10 mM Tris, 10 mM EDTA, 1 mM NaCl, 5 % SDS). Blood samples were also used to prepare thin blood smears. DNA was isolated from all samples within 2 weeks using the QIAGEN Biosprint 96 System following manufacturer's guidelines. DNA was also isolated from fecal samples using the QIAmp DNA Stool Mini Kit.

All extracted samples were subjected to a nested PCR using primer sets DW2/DW4 and DW1/DW3. These primers target approximately 600 base pairs (bp) of the mitochondrial cytochrome *b* gene (cyt*b*) of the parasites (Martinsen et al. 2006). In case of DNA degradation in the

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Table 1 Wild bird species sampled at the National Zoological Park, Washington, D.C. for malaria parasites and screening results based on parasite amplification from blood

Bird species	N birds infected/ N screened	Parasite genus
American goldfinch (*Carduelis tristis*)	1/5	*Plasmodium*
Carolina chickadee (*Poecile carolinensis*)	0/1	
Carolina wren (*Thryothorus ludovicianus*)	0/1	
Downy woodpecker (*Picoides pubescens*)	0/1	
Eastern towhee (*Pipilo erythrophthalmus*)	1/1	*Plasmodium*
Gray catbird (*Dumetella carolinensis*)	1/2	*Plasmodium*
House sparrow (*Passer domesticus*)	4/7	*Plasmodium*
Indigo bunting (*Passerina cyanea*)	1/1	*Plasmodium*
Northern cardinal (*Cardinalis cardinalis*)	6/6	*Plasmodium*
Song sparrow (*Melospiza melodia*)	2/3	*Plasmodium*
Tufted titmouse (*Baeolophus bicolor*)	4/5	*Plasmodium*
White-breasted nuthatch (*Sitta carolinensis*)	0/1	
White-eyed vireo (*Vireo griseus*)	0/1	
White-throated sparrow (*Zonotrichia albicollis*)	15/21	*Parahaemoproteus*

fecal samples, we also attempted to amplify parasite DNA from the fecal samples using primers PrCy1F and PrCy1R, which target a smaller (125 bp) cyt*b* gene fragment (PrCy1F: GCTTTAAAAATACCCTTCTATCCAA; PrCy1R: GCATTATCTGGATGTGATAATGGTA). Both of these primer sets have proven successful in the amplification of a diversity of avian malaria parasites including those from museum specimens (Martinsen et al. 2008; Fleischer et al. unpubl.). To ensure successful isolation of DNA from the fecal samples, we attempted host DNA amplification from a random subset (N = 15) of the samples using primers Cytb2 and CytbS2H, which amplify 121 bp of the cyt*b* gene from a wide range of birds (Fleischer et al. 2006). For both extraction and PCR protocols we used negative controls. PCR products were visualized by gel electrophoresis, purified using ExoSAP-IT, and sequenced on an ABI 3100 Sequencer. Using SEQUENCHER version 5.0, sequences were edited and aligned.

By nested PCR of blood samples from 56 birds of 14 species (Table 1), we found 35 birds to be positive for malaria parasite infection (prevalence of 63 %). Using the same nested PCR protocol for the fecal samples, no samples were found to be positive. Using a second PCR protocol on the extracted fecal samples, one that targets a smaller (125 bp) fragment of parasite DNA, we also found no positive infections. All negative controls were negative.

From the positive infections, we documented a diversity of malaria parasites of the genera *Plasmodium* and *Parahaemoproteus*. Twenty birds were infected by parasites of the genus *Plasmodium* and 15 by parasites of the genus *Parahaemproteus.* All positive infections were confirmed by light microscopy of blood smears. We successfully amplified and sequenced bird DNA from fecal samples isolated by both extraction procedures indicating successful DNA isolation from fecal material.

Unlike previous studies in apes and humans that find feces suitable for malaria parasite screening by standard PCR methods, we found this not to be the case in birds (Kaiser et al. 2010; Liu et al. 2010). We were unable to amplify both medium (600 bp) and short (125 bp) fragments of malaria parasite DNA from the feces of infected birds. This holds true for a diversity of malaria parasites of two genera (*Plasmodium* and *Parahaemoproteus*). Our inability to successfully amplify parasite DNA from bird feces may be due to differences in the composition of bird feces versus mammal feces, including the numerous compounds capable of degrading DNA and inhibiting enzymatic reactions, or the differential inclusion of erythrocyte remnants in avian versus mammalian feces. Further studies with captive birds are needed to determine if detectable amounts of parasite DNA are not shed into bird feces or if the parasite DNA is degraded in some way. Our results indicate that sampling of blood and/or other tissues is still necessary to properly diagnose malaria parasite infection in wild bird populations.

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