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Lung fluke (*Paragonimus africanus*) infects Nigerian red-capped mangabeys and causes respiratory disease

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ABSTRACT

Eggs of the lung fluke genus *Paragonimus* were detected in red-capped mangabeys (*Cercocebus torquatus*) in Nigeria. We assess the role of these primates as potential sylvatic hosts and the clinical effects of the parasite on monkeys. DNA sequenced from eggs in feces were 100% identical in the ITS2 region to *Paragonimus africanus* sequences from humans in Cameroon. *Paragonimus*-positive monkeys coughed more than uninfected monkeys. Experimental de-worming led to reduction in parasite intensity and a corresponding reduction of coughing to baseline levels in infected monkeys. This report provides the first evidence of *Paragonimus* sp. in *C. torquatus*, of *P. africanus* in Nigerian wildlife, and the first molecular evidence of the parasite in African wildlife. Coughing, sometimes interpreted as a communication behavior in primates, can actually indicate infection with lung parasites. Observations of coughing in primates may, in turn, provide a useful mechanism for surveillance of *Paragonimus* spp, which are re-emerging human pathogens, in wildlife reservoirs.

1. Introduction

Paragonimiasis is a food-borne illness of the lung caused by trematodes of the genus *Paragonimus*. Humans can become infected with this lung fluke after consuming raw or undercooked freshwater crustaceans. Before infecting mammalian hosts, *Paragonimus* species require a snail as the first intermediate host, and a freshwater crab or crayfish as the second intermediate host. In mammalian definitive hosts, the infective metacercariae excyst in the duodenum and migrate to the lungs, causing pulmonary paragonimiasis, with respiratory symptoms (e.g. coughing) that mimic tuberculosis (Toscano et al., 1995). Ectopic paragonimiasis occurs when flukes migrate internally, causing damage to muscles and organs, including the brain (cerebral paragonimiasis) (Blair, 2014). *Paragonimus* spp. infect more people globally than any other foodborne trematode, and infections cause an estimated 196,710 disability adjusted life-years (Fürst et al., 2012). These estimates do not account for infections in Africa.

Paragonimus spp. is best known from Asia and Latin America (Fürst et al., 2012). However, two known species, *Paragonimus africanus* and *P. uterobilaterlis*, infect humans in Africa (Blair, 2014). *Paragonimus uterobilateralis* is considered the principal causative agent of paragonimiasis in Nigeria (Aka et al., 2008). The epidemiology of *Paragonimus* sp. infections in Nigeria is tightly bound to post-colonial history. Prior to the Biafran war in Nigeria (1967–1970), paragonimiasis was known only from a handful of cases (Nnochiri, 1968; Nwokolo, 1964). During the war, food shortages and limited access to cooking facilities led to increased consumption of inadequately cooked or raw crab, and cases of paragonimiasis increased dramatically (Nwokolo, 1972). Human infections nearly disappeared again after the war, until recent surveys revealed unexpected high prevalence (up to 13.2%) in communities in the Southeast part of Nigeria (Aka et al., 2008). The epidemiology of *Paragonimus* sp. in Nigeria differs from that in Cameroon, where the disease has a longer history of endemicity due to the cultural practice of eating raw crabs in some areas (World Health Organization, 1995).

The African civet (*Viverra civetta*) is considered the natural host of *P. uterobilateralis* in Nigeria (Voelker and Sachs, 1974), with the swamp mongoose (*Atilax paludinosus*) and domestic dog (*Canis*

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familiaris) harboring the parasite in Cameroon and Liberia, respectively (Voelker and Vogel, 1965). *Paragonimus africanus* has a broader host range, infecting the mongoose (*Crossarchus obscurus*), palm civet (*Nandinia binotata*), drill monkey (*Mandrillus leucophaeus*), potto (*Perodicticus potto*), and domestic dog (*C. familiaris*) in Cameroon (Voelker and Vogel, 1965; Sachs and Voelker, 1975). The intermediate and definitive hosts of *P. africanus* range throughout the contiguous forest of southeastern Nigeria bordering Cameroon (Kingdon, 2005; Abraham and Akpan, 2011), suggesting that *P. africanus* could be more widely distributed than is currently appreciated.

We report the discovery of *Paragonimus* sp. eggs in red-capped mangabeys (*Cercocebus torquatus*) in Nigeria. We sequenced *Paragonimus* sp. DNA directly from eggs in feces to identify it to species and compare it to parasites reported in human populations. We also made observations of primate hosts for clinical signs of infection. Finally, we examined clinical observational data prior to and following treatment of the study population with anthelminthic drugs. We use this information to assess the presence of *Paragonimus* sp. in red-capped mangabeys in Nigeria, the role of these primates as potential hosts, and the clinical effects of the parasite on monkeys.

2. Materials and methods

Between May and August 2012, we collected fecal samples and recorded coughing opportunistically from a group of 49 [23 adults/sub-adults (≥3 yo) and 12 juveniles (<3 yo)] individually identifiable red-capped mangabeys that lived in a 1-ha open topped forest enclosure within the natural home range of the species (Fig. 1). The population was provisioned daily, but also had access to wild foods within the enclosure. The animals had access to water *ad libitum*, from a natural stream that ran through the enclosure. They were exposed to natural predators (e.g. snakes and birds of prey) and parasites. All animals were rescued from the bushmeat and pet trades in Nigeria as young juveniles, or were captive-born. Thirteen individuals were moved from a sancutary to the open-topped enclosure in 2004 as part of the rehabilitation and release program of the Centre for Education, Research and Conservation of Primates and Nature (CERCOPAN), and the remaining 36 individuals were born in the enclosure.

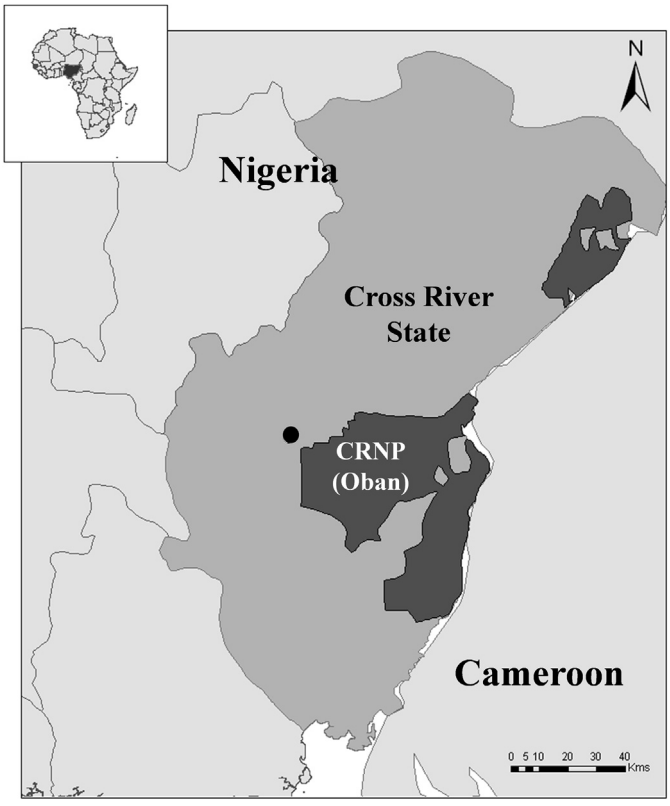

Fig. 1. Map of collection site. Map shows the location of the study population (*black circle*) relative to the Oban Division of Cross River National Park (CRNP), in Cross River State, Nigeria.

For 30 days in late May and June 2012, we collected triplicate fecal samples from each individual. Then, in late June 2012, the population was treated for *Paragonimus* sp. via orally administered praziquantel (approximately 20 mg/kg for three consecutive days). We collected subsequent triplicate fecal samples from each individual over 30 days post-treatment, for a total of 294 samples (147 pre-treatment and 147 post-treatment). Over the same time periods (30 days pre-treatment and 30 days post-treatment), we recorded all observed instances of coughing between 6:00 and 17:00 daily. The Institutional Animal Care and Use Committee at University of Wisconsin, Madison approved all research activities (protocol v1490).

Fecal samples were collected from known individuals immediately following defecation, stored temporarily in plastic bags, and fixed within 2 h of collection. We removed two aliquots from each sample for preservation of gastrointestinal parasite eggs and DNA separately. One aliquot was fixed in 10% formalin for microscopic analysis, and the other in RNA*later*® nucleic acid stabilizing solution for genetic analysis. Samples were transported to the University of Wisconsin, Madison following all applicable import, export, and International Air Transport Association regulations. One gram of formalin-preserved feces was concentrated by sedimentation and examined microscopically at X10 and X40 magnification (Greiner and McIntosh, 2009). We calculated prevalence (percent of individuals infected) as number of individuals shedding eggs divided by the total number of individuals examined, and we approximated mean and median intensity of infection (number of eggs per gram (epg) of a particular parasite species in the feces of a single infected host) (Bush et al., 1997; Greiner and McIntosh, 2009). We calculated pre- and post-treatment intensity by taking the average epg of triplicate samples for each individual. We compared *Paragonimus* sp. prevalence and intensity to host characteristics and rates of coughing using Fisher's exact test, Mann–Whitney test, and Spearman rank correlation. We then compared parasite intensity and coughing rates pre- and post-treatment using paired Wilcoxon rank sum test. For all pre- and post-treatment comparisons, we used one-tailed tests under the directional hypotheses that coughing frequency would be positively associated with parasite infection.

We extracted DNA from 150 mg of the fecal sample with the highest egg count (1,067 epg) using the Zymo ZR Fecal DNA Mini-Prep Kit (Zymo Research Corporation, Irvine, CA, USA), following the manufacturer's protocols. PCR and nucleotide sequencing were performed on the internal transcribed spacer 2 region (ITS2) using primers 3S (5′-CGGTGGATCACTCGGCTCGT-3′) and A28 (5′-CCTGGTTAGTTTC TTTTCCTCCGC-3′), previously used to amplify *P. africanus* (Nkouawa et al., 2009). PCR was performed using Phusion High-Fidelity PCR mastermix (New England BioLabs, Ipswich, MA), and cycled in a BioRad CFX96 platform (Bio-Rad Laboratories, Hercules, CA, USA) with the following cycling parameters: 98 °C for 30 min; 40 cycles of 98 °C for 10 s, 55 °C for 30 s, 72 °C for 90 s; and a final extension at 72 °C for 10 min. Amplicons were electrophoresed on an agarose gel stained with ethidium bromide and then purified using the Zymoclean Gel DNA Recovery Kit (Zymo Research Corporation, Irvine, CA, USA). Amplicons were sequenced