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Plasmodium Infection of Non-Human Primates Living in Wildlife Sanctuaries Located in Cross River State, Southern Nigeria

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ABSTRACT:
Objective: This study on *Plasmodium* infection of non-human primates living in wildlife sanctuaries located in Cross River State, Southern Nigeria was aimed to determine the presence of malaria parasites in these primates. **Methods**: Microscopy and Molecular methods were used to characterise the species of these parasites from April 2013 to June 2014 during the period of the rainy and dry season. Blood samples were obtained from 41 captive primate species, *Mandrilus leucophaeus, Cercopithecus species,* and *Pan troglodytes from* wildlife sanctuaries in Cross River State. **Results**: Prevalence of *P. falciparum* malaria amongst the non-human primates by polymerase chain reaction method was 15% and 7% in *Mandrilus leucophaeus* and *Cercopithecus species* respectively. **Conclusion**: *P. falciparum* infection of the primates implies that given the enabling environment and existence of an efficient vector, these non-human primates could be sources of infection to humans and reservoir for residual malaria in the future.

Keywords: Malaria, *Plasmodium*, primates, PCR, microscopy

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Author's contributions: This work was carried out and approved in collaboration between all the authors who take responsibility for its intellectual contents, accuracy and integrity. OAO designed the study; AAAA sourced for funding; OAO, AAAA, MFU wrote the protocol; OAO, AAAA contributed in literature search; OAO did Lab experiments; OAO did the statistical data analysis; OAO, AAAA, MFU contributed in discussions; OAO drafted the manuscript; AAAA, MFU supervised the study; OAO, AAAA, MFU wrote the final manuscript; OAO, AAAA, MFU proofread the final version for publication.

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INTRODUCTION

Recent WHO reports have shown a decline in the incidence of malaria and deaths attributable to the disease globally due to the sustained scale-up of malaria control interventions in malaria endemic countries (1). This could mean that the "Malaria elimination programmes" of malarious countries are yielding positive results. According to WHO, deaths caused by malaria have reduced by 42% and 49% globally and in Africa respectively (1). Despite these gains in global malaria control efforts, between one to three billion people worldwide are still at risk of malaria infection (1). It is interesting to note that most studies done in Africa show that the four human plasmodium species namely *P. falciparum*, *P. ovale*, *P. malaria* and recently *P. vivax* are responsible for malaria infection (2-5). In southeast Asia (SEA), the simian parasite *P. knowlesi* has now been recognized as the fifth malaria species responsible for clinical malaria infections in humans (6). *Plasmodium knowlesi* is suspected to be responsible for a significant proportion of hospitalization caused by malaria in some Southeast Asian countries, (6-8).

In Africa, studies have reported the presence of some simian malaria such as *P. gaboni*, *P. rechienowi* and human malaria species such as *P. falciparum* and *P. malariae* in the faeces and blood of wild primates (9, 10).

There has been a concern for the incidence of residual malaria in areas where malaria has been eliminated or nearly eliminated (11). This is because most control programmes such as Long Lasting Insecticide Treated Net (LLITN) and indoor Residual Spraying (IRS) target mosquitoes that feed and rest indoor after feeding on humans (12-14). The implication of this is that *Anopheles species* that feed outdoor and rest outdoor after blood meal will not be affected by these control methods (15). Thus when these *Anopheles* mosquitoes are the species that can feed on both humans and animals, they will be able to maintain malaria transmission between the two hosts. This can occur if the mosquito permits the parasite to complete its sporogony and survives long enough to bite these hosts (15). As it is being reported in studies from SEA, reduction in active malaria transmission as a result of effective control programme has brought with it the challenge of residual malaria (11). Residual malaria is defined as persistent malaria transmission after the successful implementation of effective control programmes (15). In some countries such as Nigeria, the LLITN is the control method adopted by National Malaria Control programmes.

A review of current global literature on this subject shows that no research on malaria infection of primates living close to humans has been reported from Nigeria in contrast to other neighbouring countries such as Cameroon and Gabon (9, 10). A report of the increase in clinical illness from *P. knowlesi* infections is not new in other regions (16). In SEA, the people who are reported to be most at risk of *P. knowlesi* infections are those who live close to forest fringes and those who keep the primates as pets. The most widely distributed malaria vectors in the study area are the species of the *A. gambiae sensu Latus* such as *A.gambie s.*s and *A.colluzzi* (17-18) and *A.arabiensi* (19). It has become necessary to assess the status of simian malaria in areas like Cross River State where malaria morbidity and mortality are still relatively high (20).

This study aimed to determine the species composition of the genus *Plasmodium* in non-human primates living in wildlife sanctuaries in Cross River State Nigeria. Here we used multiplex PCR to determine the presence of *Plasmodium* species in *Pan troglodytes* (Chimpanzee), *Mandrillus*

leucophaeus(drill monkeys) and *Cercopithecus* species (potty-nosed monkeys) living in these sanctuaries.

MATERIAL AND METHODS

Ethical statement: Ethical approval for the use of animals in this study was obtained from the Ethical Review Committee/ of the Cross River State Ministry of Health. Also, permission and letters of support were obtained from the conservator General of the Nigeria Wildlife National Park, Cross River State Forestry Commission, Directors of Pandrilus, Nigeria, and Centre for Education, Research and Conservation of Primates and Nature (CERCOPAN), Nigeria.

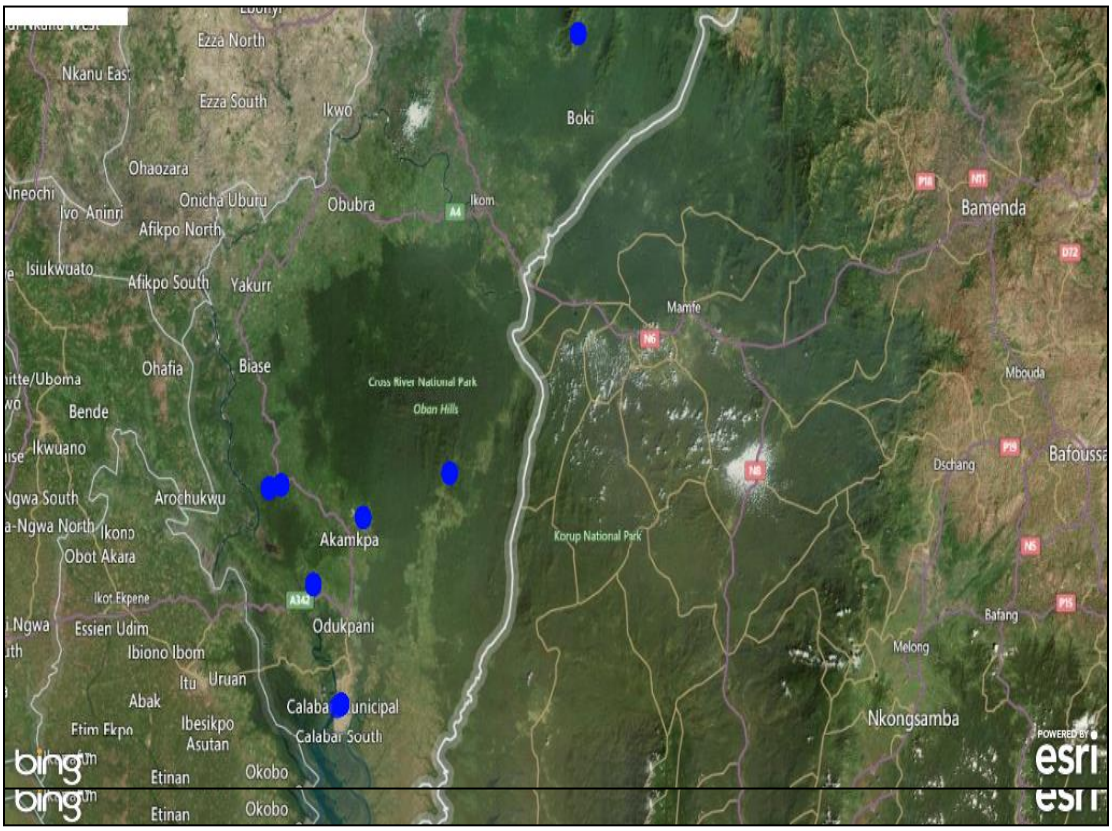

Figure 1: Geographic Positioning System (GPS) Location of areas in Cross River State, where data was obtained (blue circles are locations where sampling was done). Calabar municipal, Latitude 4.977^0, Longitude 8.334^0, Altitude 135m; Drill Ranch in Afi Mountain Wildlife Sanctuary; Latitude 6.2999^0 and Longitude 8.9977^0, Altitude 300m and Rhoko forest Latitude 4.9714 Longitude 8.3216 Altitude 135m

Study setting:

The rain forest belt of Nigeria where Cross River State is located has a fairly large population of non-human primates including endangered species such as *Mandrilus leucophaeus* (drill monkeys) and *Cercopithecus* species (putty-nosed monkeys) which are found only in Nigeria and its Cameroon bother. Wildlife sanctuaries where these primates are protected are located within protected forests of Cross River State close to where humans dwell as well as in Calabar, the capital city of Cross River state.

Study populations:

Forty-one primate made up of 7 *Pan troglodytes*, 20 *Mandrilus leaucophaeus*, and 14 *Cercopithecus* species living in the sanctuaries that required veterinary care were consecutively included into this study. The choice of this category of primates was to minimize discomfort, and pain in the animals as recommended by The Guidelines for Use of Animals in Neuroscience and Behavioral Research that encourages humane treatment of animals (22). Hence, no animal was anesthetized because of this study.

Sample size calculation for non-human Primates:

Determination of the minimum sample size for the primates was as described in "The Guidelines for the Care and Use of Mammals in Neuroscience and Behavioural Research" (22). Using the formula "n= logβ/logp, where 'p' is the proportion of the animals not infected (100-proportion of animals infected). β is the probability of committing a Type II error at 0.05". The sample size was based on the prevalence of 12% which was the proportion of primates with malaria infections in Cameroon (9). Therefore, "n" of primates at 95% chance of detecting malaria infection, is: log0.05/log0.88= 23. However, 41 primates were finally tested for malaria including 10 samples from the archive of Pandrillus, Nigeria.

Routinely, sick primates and those in quarantine sometimes require laboratory investigation for their management. At the wildlife sanctuaries, blood samples are collected into bottles containing ethylenediaminetetraacetic acid (EDTA) from primates that require veterinary care for Laboratory investigations. During such procedures, the veterinarian collects blood simultaneously from consecutive primates for this study on filter paper and microscope slide.

Four drops of blood were collected for malaria microscopy (thin and thick films) and another four drops on filter paper for Polymerase Chain Reaction (PCR) assay. A drop of blood for a thin smear and three drops for a thick smear were transferred to a grease-free slide and allowed to dry. After drying, the blood smear was stained in 3% Giemsa stain for 45minutes. Two hundred microlitre of blood was transferred to a piece of Whatman™ 3MM 1 Chromatography paper (GE Healthcare UK Limited) and dried, enveloped, and preserved in silica gel for polymerase chain reaction (PCR) technique. The filter papers were dried before sealing each sample separately in an envelope that contains "silica gels" as desiccants. Dry blood spots were kept at room temperature until ready for processing at the United States Naval Medical Research Unit No 3 in Cairo and its Ghana detachment

Molecular (PCR) method

DNA extraction of blood: Molecular investigations in this study were carried out at the United State Naval Medical Research Unit No .3 (NAMRU-3), Cairo Egypt and NAMRU-3 Ghana detachment at the Noguchi Memorial Institute for Medical Research, University of Ghana. DNA was extracted from filter paper dry blood spots from primates using the QIAamp DNA Mini kit by QIAGEN (Germany) according to the manufacturer's instruction. Malaria infection was determined by multiplex PCR amplification of *Plasmodium* small subunit 18S ribosomal RNA (SSUrRNA) as described in previous studies (23, 24). The specific forward primer used for each species were *P.falciparum* (PF, Sequence, 5^1-AAC AGA CGG GTA GTC ATG ATT GAG 3^1) , P. *vivax* (PV Sequence, 5^1 CGG CTT GGA AGT CCT TGT 3^1, *P. ovale* (PO Sequence, 5^1 CTG TTC TTT GCA TTC CTT ATGC 3), *P. malariae* (PM Seguence, 5^1 CGT TAA GAA TAA ACG CCA AGCG 3), and Reverse primer (R Sequence, 5^1 GTA TCT GAT CGT CTT CAC TCCC 3^1), that is conserved in all four species (23). To detect the presence of *Plasmodium* species other than the four human species, all negative DNAs were re-amplified using genus-specific Plasmodium Universal primer (rPLU) for Plasmodium genus amplification $rPLU_5$(5′-CCTGTTGTTGCCTTAAACTTC-3′) and$rPLU_6$(5′-TTAAAATTGTTGCAGTTAAAACG-3′) as earlier explained by Snounou(25),.These primers were based on the sequences of the small subunit 18S ribosomal RNA genes(5). Products that were amplified were sequenced and compared with the available database. Results were scored independently by two analysts; all nonspecific amplifications were repeated for confirmation.

RESULTS

This study screened 41 primates comprising of 7 *Pan troglodytes*, 20 *Mandrilus leaucophaeus*, and 14 *Cercopithecus* species for malaria parasite infections using light microscopy and molecular techniques.

Of the 41 primates that were tested for the presence of malaria parasites, only four (9.8%) were positive *for P.falciparum* by PCR, while one (2.4%) primate was positive by microscopy (Table 1). The result obtained shows that 3 (15%) of the *Mandrillus leaucophaeus* and and 1 (7.1%) of the *Cercopithecus* species were positive for *P.falciparum* respectively (Figure 2). However, only the *Cercopithecus* species was positive for *P.falciparum* by microscopy. None of the 7 chimpanzees tested was positive for malaria parasites.

Table1. Prevalence of *Plasmodium species* infection amongst primates examined by Microscopy and PCR

Species of Primates	No. Examined	No. detected by Microscopy (%)	No. detected by PCR (%)
Pan troglodytes	7	0	0
Mandrilus leucophaeus	20	0	3 (15)
Cercopithecus species	14	1 (7.1)	1 (7.1)
Total	41	1(2.4%)	4 (9.8)

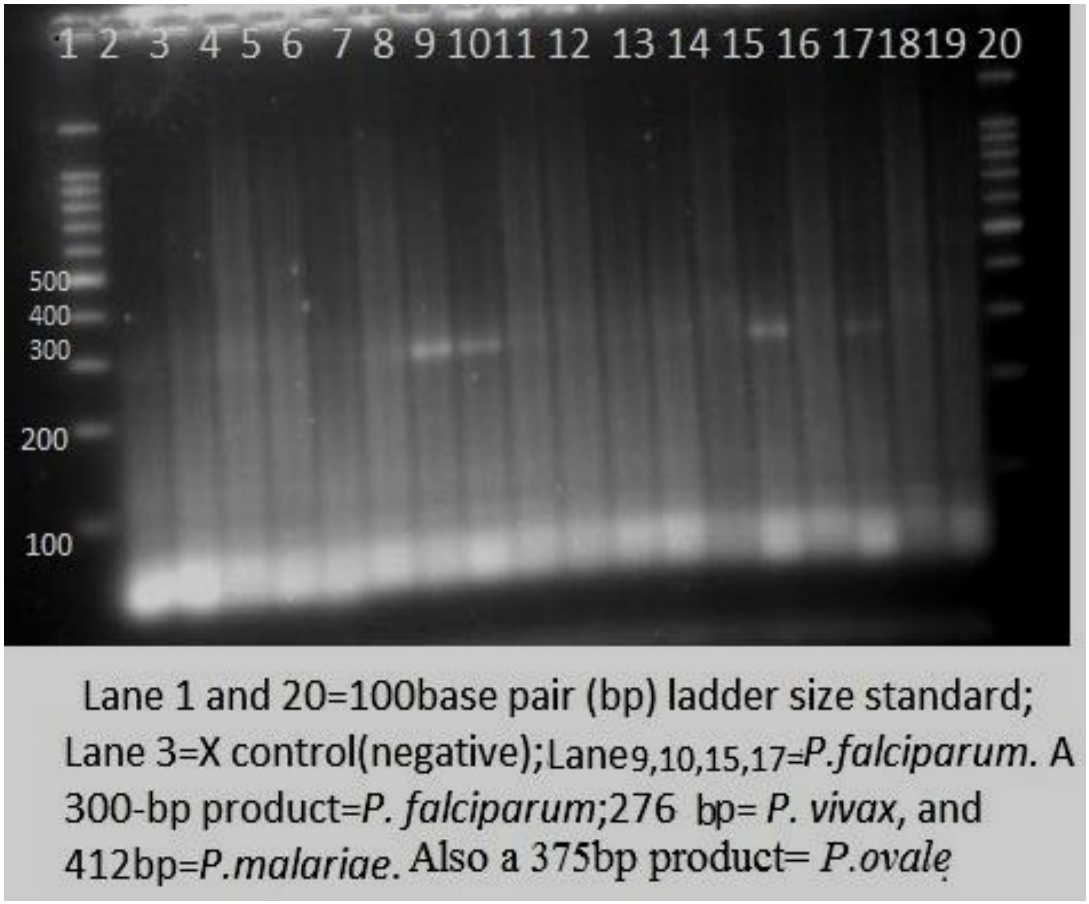

Figure 2: Multiplex PCR of primates blood showing positive results for *P.falciparum*

DISCUSSIONS

Plasmodium falciparum was detected in three drill monkeys (*Maandrillus leucophaeus*) and one *Cercopithecus species* This is similar to findings from other studies recently carried out in Cameroun and other African countries (26-28). It was previously believed that *P. falciparum* was restricted to infecting only humans (26,27). The implication of detecting human malaria parasites in these non-human primates is that given the enabling environment and existence of an efficient vector, primates infected with simian malaria could be sources of infection to humans (29). Also, the susceptibility of these primates to *P. falciparum* shows that they can become reservoir (30) for residual malaria in the future after the successful elimination of the parasites by the National Malaria Control Programmes (NMCP). In some regions where extensive programmes aimed at eliminating malaria have been ongoing for years; this goal has been difficult to achieve because malaria control programmes were targeting mostly vectors that feed on humans and around human dwellings (12,28)

Results of vector mapping carried out at the same location and period could not explain the vector likely to be responsible for transmitting the *P.falciparum* to the primates (17). Most (98%) of the mosquitoes caught in that study were *Anopheles gambiae s.l.* of which. *Anopheles gambiae s.s* were 75% (17). Another report from the same location showed that over 80% of *Anopheles* species caught were *A. gambiae sensu Latus* of which 65% were *A.gambiae s.s* (18). Since *A.gambiae s.s.* is anthropophilic and endophagic, it is probably not an effective vector for transmission of malaria between humans and apes because there is no evidence that this species feeds on any other primate apart from humans (30).

There may be several reasons which were unknown to us why this study did not detect simian malaria in any of the primates tested. Interestingly, one of the drill monkeys positive for malaria in this study had been positive for malaria in the past and was treated with ACTs. Other studies have also detected *P. falciparum* in gorillas, chimpanzees, and monkeys living in wildlife sanctuaries (26-28). It is believed that sources of infection were most likely from humans through the bites of mosquitoes not commonly encountered in their natural habitat (31). This is because *P. falciparum* infection has only been found in wild primates living in captivity (32,33). Such primates, therefore, constitute an increasing pool of non-human sources of *P. falciparum* infection transmitted by outdoor biting mosquitoes.

The incidence of residual malaria is now on the increase in areas where malaria had previously been eradicated (11). One of the reasons given for this is that mosquitoes have changed their biting habits so that endophagic and endophilic species now bite outdoor and may also rest outdoor to avoid making contact with the insecticides (12-14). If the plan of sub-Saharan African countries to eliminate malaria transmission must succeed, effective control programmes should not only target indoor biting and resting mosquitoes but also those that can bite both humans and animals outdoors. There is a need for a large study that will investigate the interaction between humans, non-human primates living in captivity close to human dwellings, and disease vectors. This may provide information to determine whether captive primates are at the risk of contracting malaria and other pathogens such as tuberculosis and measles which in turn may endanger humans that have contact with them (27).The main limitation of this study was that due to limited resources, the *P.faliciparum* species detected were not further subjected to genomic sequencing to

determine the true origin of the *Plasmodium* species.

In conclusion, this was the first study in Nigeria to the best of our knowledge to investigate malaria infections in captive non-human primates living near human dwellings. It observed *Plasmodium falciparum* infections in 9.8% of the primates screened by multiplex PCR.

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