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Short Communication

First report of malaria parasites in water buffalo in Nepal☆

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ABSTRACT

We present the first molecular-based report on ungulate malaria parasites from water buffalo in Nepal. Fifty-six blood samples were collected from different groups of water buffalo (wild, feral, and domestic) and PCR assays were conducted using *Plasmodium* spp. *cytb* specific primers. Two positive cases were detected, one each from feral and domestic individuals. Complete mitochondrial genome sequence (5987 bp) was obtained and examined for nucleotide variations. Sequence analysis revealed identity with type II water buffalo malaria parasites, reported previously, with one A to T nucleotide difference at position 5344. Prevalence, as well as possible economic impacts of water buffalo malaria, should be determined on a wider set of samples from buffalo across Nepal.

1. Introduction

Nepal is an agricultural country with much of the population still engaged in agriculture and livestock farming. Livestock farming in Nepal suffers economic losses due to protozoan pathogens such as *Babesia*, *Theileria*, and *Trypanosoma*; parasites which infect cattle, water buffalo, pigs, goats, and sheep. The locally available diagnostic techniques to identify protozoan parasites are largely based on microscopic or serological examinations (Maharjan and Mishra, 2006). Microscopic diagnostics are not effective techniques, because they lack sensitivity and specificity (Böse et al., 1995). The serological techniques based upon ELISA, despite being highly sensitive at detecting parasitic infections in animals, fail to differentiate between current and prior infections. PCR-based diagnoses have high sensitivity and specificity in detecting and identifying parasite species, but in Nepal these techniques are not commonly used.

Malaria disease is caused by intracellular protozoan *Plasmodium* parasites, transmitted by the bite of mosquito vectors. In humans, malaria is caused by five different species which cause a great burden of global morbidity and mortality. Apart from humans, malarial parasites have been known to infect a wide range of hosts including non-human primates, rodents, bats, ungulates, birds, and reptiles (Cox, 2010; Garnham, 1966). The malaria parasite in domestic water buffalo was first reported through microscopic observation in India, and named *Plasmodium bubalis* by Sheather Sheather (1919). Since then sporadic cases of *P. bubalis* have been described based upon microscopy descriptions, mostly in immunocompromised water buffalo (Kolte et al., 2002). In 2016 Templeton et al. reported molecular-based surveillances of malaria parasites in water buffalo in Thailand and Vietnam and found up to 45% of the examined animals were infected with malaria parasites (Templeton et al., 2016). They found that there were two distinct *Plasmodium* mitochondrial DNA sequences and provisionally designated them as types I and II. Quantitative PCR analysis revealed that the type I parasite sequence was present at higher densities than type II. Type I and II sequences were deposited as *Plasmodium bubalis* (accession number LC090213) and *Plasmodium* sp. ex *Bubalus bubalis* (LC090214), respectively.

The Koshi Tappu Wildlife Reserve (KTWR) is located in the eastern

☆ Sequence obtained in this study was deposited in GenBank with the accession number MK518339.

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lowland Terai region of Nepal. The reserve was established in 1976, primarily to protect the last remaining Nepalese population of an endangered Asiatic wild buffalo (*Bubalus arnee*) (Hedges et al., 2008), the wild progenitor of domesticated water buffalo locally called 'Arna' (*B. arnee*). It is the smallest wildlife reserve in Nepal with an area of 176 km^2. In a recent census 432 individuals were recorded (KTWR, 2016); 182 adult females, 120 adult males, and 130 calves. Wild buffalo co-habit the reserve with highly backcrossed feral animals thought to have been released in the area around 70 years ago (Dahmer, 1978). KTWR is surrounded by heavy settlements thus buffalo protected inside the reserve frequently share grazing area with domestic animals. Wild buffalo in KTWR are under threats due to habitat loss, genetic introgression from feral backcrosses, and transfer of diseases from domestic buffalo. This species is considered highly endangered globally (Hedges et al., 2008), thus maintaining healthy populations will have greater advantages in the genetic improvement programs of domestic stocks.

In the present study, nested PCR assays were performed to detect the presence of malaria parasites in water buffalo (wild, feral, and domestic) in Nepal, using oligonucleotide primers specific for the *Plasmodium* mitochondrial cytochrome *b* (*cytb*) sequence.

2. Materials and methods

2.1. Sampling site

The study was conducted at water buffalo sites in Koshi Tappu Wildlife Reserve (KTWR) and surrounding villages (Bhagalpur, Kushaha, Uttriduvan, Dakshitidovan, and Haripur). KTWR lies along the bank of the Sapta-koshi River in the south-eastern Terai region of Nepal (Fig. 1). A total of 56 water buffalo are included in the present study. The buffalo were classified into three groups as wild (n = 16), mixed wild/domestic ancestry (referred as feral; n = 23), and domestic (n = 17). This classification is based on the widely used phenological and behavioural characteristics of buffalo (Dahmer, 1978; Flamand et al., 2003; Heinen and Singh, 2001). Out of 56 water buffalo, 32 were female and 24 male. The ages of the buffalo were recorded for 38 samples, which ranged from 0.5 months to 15 years. The ages of the wild (n = 15), feral (n = 1), and domestic (n = 2) buffalo are not known (Supplementary Table 1). Blood samples from three wild buffalo (*B. arnee*) in Central Zoo, Lalitpur, and 13 wild buffalo (*B. arnee*) from KTWR were taken during their translocation to Chitwan National Park in January to February 2017 (winter season). The remaining 40 blood samples were collected from feral and domestic buffalo (*B. bubalis*) in June and July 2017 (rainy season). Blood samples (5 mL) were collected from the jugular vein from feral and domestic buffalo chosen randomly without considering age and sex. All blood samples were originally collected for a population genetic study of wild buffalo of KTWR (manuscript under review) and the approval of translocation was granted by the Nepal Government, Ministry of Forestry and Soil Conservation, Department of National Parks and Wildlife Conservation.

2.2. DNA extraction, PCR, and sequencing

Genomic DNA was extracted from blood samples using a commercial kit (QIAGEN DNeasy Blood and Tissue kits, QIAGEN, Germany) according to the manufacturer's protocol, and stored at 4 °C.

PCR assays using *Plasmodium cytb* gene specific primers were conducted as described (Martinsen et al., 2006). Briefly, the *cytb* gene of *Plasmodium* was amplified by nested PCR using the universal primers for *Plasmodium* DW2 (TAATGCCTAGACGTATTCCTGATTATCCAG) and DW4 (TGTTTGCTTGGGAGCTGTAATCATAATGTG) for the first round PCR. The nested PCR reaction was performed using primers NCYBINF (TAAGAGAATTATGGAGTGGATGGTG) and NCYBINR (CTTGTGGTAAT TGACATCCAATCC) as described (Abkallo et al., 2014). Both PCR rounds were performed for 40 cycles with denaturation at 94 °C for 20 s followed by annealing and extension at 62 °C for 3 min. All reactions were performed using negative controls (deionised nuclease free water), and no contamination was detected. PCR products were confirmed by separation on agarose gel electrophoresis and viewing using the InGenius bio-imaging system (Syngene UK) (Fig. 2). For purification, 5 µL of PCR product and 2 µL of ExoSap-IT Express PCR Product Cleanup (Affymetrix Inc. Santa Clara, CA, USA) were incubated at 37 °C for 4 min followed by 80 °C for 1 min. The purified products were sequenced with a BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) according to the manufacturer's protocol using

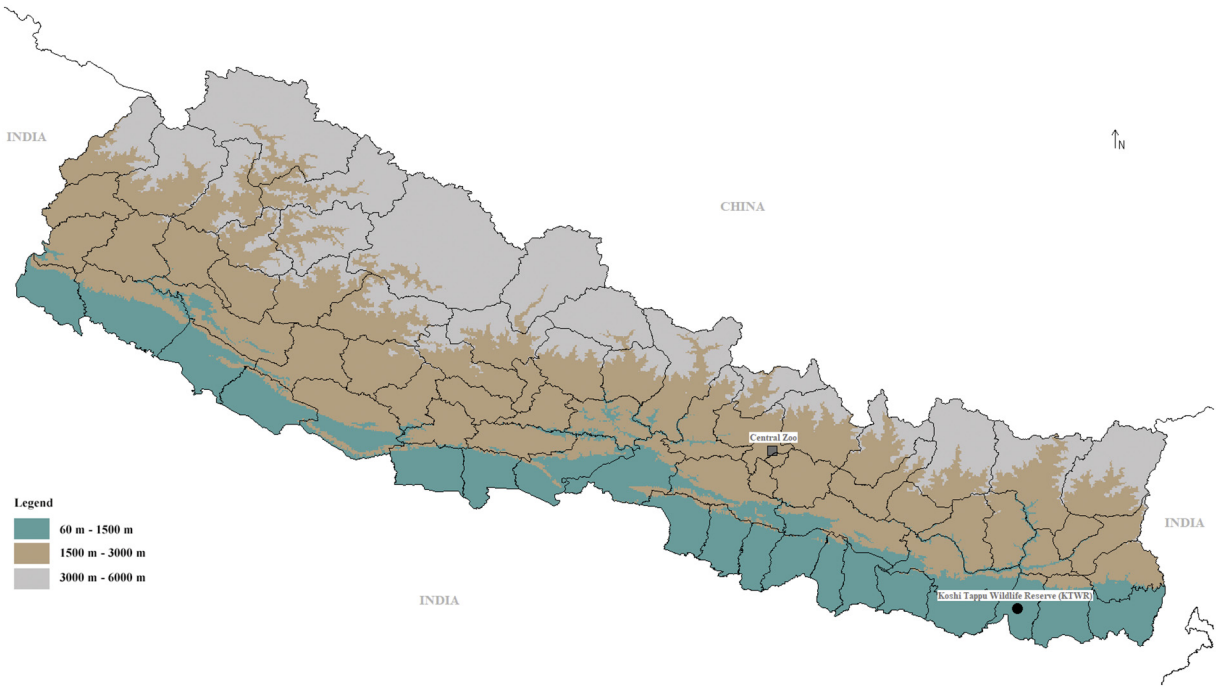

Fig. 1. Map of Nepal showing the localities from where buffalo blood samples were collected.

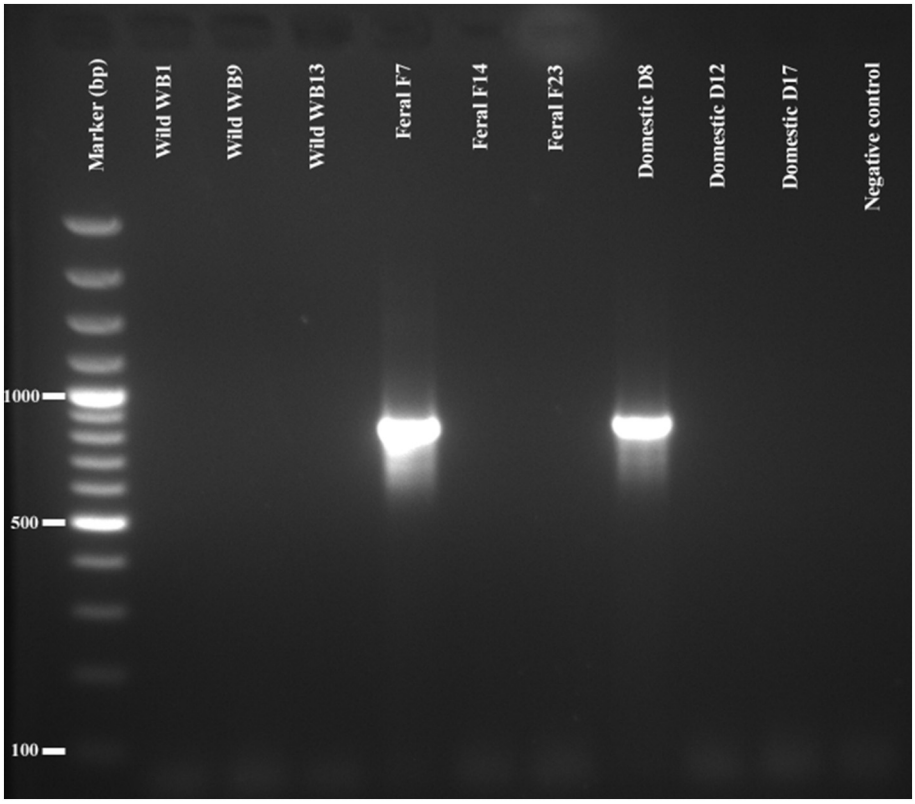

Fig. 2. Agarose gel electrophoresis showing the size of amplified products by nested PCR using primers targeting mitochondrial cytochrome *b* (*cyt*b) gene. Lane 1, DNA marker; lane 2–4, samples from wild buffalo; lane 5–7, samples from feral buffalo; lane 8–10, samples from domestic buffalo, lane 11, negative control (deionised nuclease free water).

NCYBINF and NCYBINR primers. These sequencing reactions were purified using a Big Dye Xterminator™ Kit (Applied Biosystems) and analyzed in an automated 3500XL Genetic Analyzer (Applied Biosystems). One PCR sample positive for *Plasmodium* sp. was used to obtain complete mitochondrial genome sequence using a panel of oligonucleotide primers as described (Templeton et al., 2016). Raw DNA sequence data was assembled and aligned using Sequencer 5.0 (Thompson et al., 1997) and DNA contigs were generated using forward and reverse sequences. Consensus sequences thus obtained were aligned with *Plasmodium bubalis* type I and II sequences deposited in GenBank (LC090213 and LC090214) using Bioedit v7.2.5 (Hall, 1999). Whole genome sequence of the D47 sample was deposited in GenBank with the accession number MK518339.

2.3. Ethical statements

The study was approved by the Department of National Parks and Wildlife Conservation, Kathmandu, Nepal.

3. Result and discussion

Out of 56 blood samples 16 (29%), 23 (41%), and 17 (30%) were from wild, feral, and domestic buffalo, respectively. Out of 16 wild buffalo, 13 and 3 samples were obtained from KTWR and Central Zoo, respectively. Among the buffalo, 32 (57%) were female and 24 (43%) were male. PCR assays detected 2 positive cases (4%); one was a feral (F23; 2 years old female) and the other a domestic buffalo (D47; 2 years old male). Details of demographic and molecular results are presented in Supplementary Table 1. Both positive samples were collected during the rainy season from KTWR and its surrounding villages. None of the 16 wild buffalo samples collected during the winter season from KTWR and Zoo were positive; however, further tests must be done on all existing individuals of the reserve for the confirmation of overall prevalence of the parasite. Previously, 16% (15/95) and 65% (65/144) of water buffaloes were PCR positive for malaria parasites in Thailand in 2015 and 2014, respectively; and 6% (3/49) and 5% (2/43) in Vietnam in 2013 and 2010, respectively (Templeton et al., 2016). The low frequency of the positivity in this study may be attributed to the seasonality or the mosquito vectoral capacity of each area. Approximately 48% of the Nepal human population lives in malaria endemic areas. The study site KTWR is in the lowland of the Terai region where human populations are at moderate risk for human malaria parasites. Female *Anopheles* spp. mosquitoes, which are the proposed vectors for ungulate malaria parasites, have been reported in the study areas (GON/DHS-EDCD, 2016). The distribution of *Anopheles* species varies among Nepal, Vietnam, and Thailand. In Nepal, *Anopheles fluviatilis*, and *Anopheles annularis* are the main vectors for human malaria parasites while the presence of *A. minimus* and *A. sundaicus* was reported in Vietnam and Thailand. To clarify this point, more samples from different seasons need to be analyzed.

DNA sequences of two positive PCR products (F23 and D47) amplified with *Plasmodium*-specific primers were 773 bp in length and identical. When these sequences were compared with type I and II *Plasmodium* sequences obtained from water buffalo, they show complete similarity to the type II sequence with the exception of one A to T non-synonymous substitution at nucleotide position 5344 (LC090214), a nucleotide type seen in the type I sequence (LC090213). Because this *cytb* region in type II sequences of 39 water buffalo in Thailand were identical (Templeton et al., 2016), the identified A5344T single nucleotide polymorphism in Nepal may serve as a region-specific marker. To evaluate if there are substitutions other than A5344T in *cytb*, a complete mitochondrial genome sequence (5987 bp) obtained for D47 was compared with type I and II sequences; however, no other substitutions were detected (Fig. S1). In the previous study, type II sequences were detected in 63/80 (79%) and 2/5 (40%) *Plasmodium* positive cases in Thailand and Vietnam, respectively; and type I was found in 43/80 (54%) and 3/5 (60%) positive cases in Thailand and Vietnam, respectively (Templeton et al., 2016). Based upon this prevalence the type I water buffalo malaria parasite is also likely present in Nepal, but more samples are required to evaluate its existence.

Buffalo farming is one of the major occupations in the lowland and mid-hills of Nepal. The study area KTWR stretches northward from the Nepal India border where the annual buying and selling of buffalo takes place between farmers residing across the border. Since the first water buffalo malaria parasite report from India, the prevalence of this pathogen in different areas of India has been reported by subsequent studies; such as Bareilly/Muktesar (Sheather, 1919), Nagpur (Kolte et al., 2002), and Maharashtra (Shastri et al., 1985; Shinde et al., 2005). In this context, it should be considered to quarantine water buffalo crossing the border between Nepal and India to prevent the spreading of ungulate malaria parasites.

An early study concluded that this pathogen causes only mild symptoms and animals (*B. bubalis*) were self-cured (Riaz-Ul-Hassan, 1953), although one mortality case has been observed (Templeton et al., 2016). It was not determined that the buffalo (*B. bubalis*) died due to malaria infection or if the malaria parasite was an opportunistic infection (Templeton et al., 2016). Nonetheless, the hidden pathogenesis of this pathogen as an opportunistic agent has not been evaluated. The pathogenesis in buffalo having different genetic backgrounds (wild, feral, or domestic buffalo) is also not understood. The objective of KTWR is to conserve the last remaining Nepalese population of an endangered Asiatic wild buffalo. Diagnostic assays therefore are strongly recommended to confirm the possible health impact of malaria in the wild individuals of the reserve.

This is the first report on malaria parasites in ungulates in Nepal. Passive case detection is used for diagnosis of most common parasitic diseases in domestic animals in Nepal. Vaccination is available for foot and mouth diseases, haemorrhagic septicaemia, and black fever for buffalo. Farmers regularly practice drenching for buffalo to control ticks and mites. However, farmers are unaware of malaria infection in buffalo and there are no precautionary and treatment programs for Government and relevant Government agencies.

There are few studies of malaria vector distribution in Nepal. Dhimal et al. (Dhimal et al., 2014) reported that the mosquito vectors for human malaria are abundantly found in the Terai region of Nepal during post-monsoon (rainy season) compared to pre-monsoon (dry) seasons. Similarly, human malaria parasite prevalence in Nepal is high during the rainy season compared to the dry and winter seasons (GON/DHS-EDCD, 2016). Although it was not experimentally proven, *Anopheles* spp. capable of transmitting human malaria parasites were proposed to also transmit *P. bubalis* (Garnham, 1966). Thus we assume that the seasonality of the mosquito may also affect the prevalence of *P. bubalis* in water buffalo. Although we did not detect malaria parasites from wild buffalo, perhaps because we collected blood from wild buffalo at a single time point during translocation (January–February), it is still not clear if wild buffalo are free or not from *Plasmodium* spp. infection. Further multiple sequential blood collections would improve the detection sensitivity of parasites in Nepal.

In conclusion, this is the first PCR-based surveillance of water buffalo malaria parasites in Nepal. The presence of malaria parasites in feral and domestic animals may have greater consequences in terms of ecological impact as well as the economy of farmers dependent on livestock farming. Furthermore, active surveillance is necessary for malaria parasite infection in Asiatic wild buffalo to obtain information regarding health status and mortality rates. These steps are important to develop strategies for the management and control of malaria infection especially in wild buffalos in Nepal.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vprsr.2019.100348.

Declaration of Competing Interest

There are no conflicts of interest for this work.

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