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Original article

DETECTION OF A NEW APICOMPLEXA GROUP FROM BUFFALOES IN MOSUL CITY, IRAQ

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Summary

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This study was focused on the detection of a new apicomplexan parasite (*Plasmodium* spp.) and its clinical and haematological effects during infection of domesticated water buffaloes (*Bubalis bubalis*) in Mosul city, Iraq. Although *Plasmodium* parasites of ungulates are diverse and distributed worldwide, no data are available in Iraq about any ungulate malaria, so the current investigation endeavoured to bridge this gap in the existing body of knowledge. The study included 70 cases of domesticated water buffaloes at different ages and from both sexes that were brought to the Veterinary Teaching Hospital, University of Mosul, Mosul, Iraq. The animals were from different regions of Mosul in northern Iraq. Microscopic examination was carried out on blood smears to detect *Plasmodium* parasite. The nested PCR assay was also conducted using *Plasmodium* spp. cytochrome b gene (*cytb*) specific primers to confirm the infection. Results showed the presence of *Plasmodium* parasite in 24.28% (17/70) of cases. *Plasmodium bubalis* was detected by PCR in three cases from 11 buffaloes. Among infected buffaloes, the symptomatic cases of malaria were 64.5%, while only 35.5% were asymptomatic (occult) cases. Moreover, fever in 54% of cases, paleness of the mucous membranes in 36% of cases, and recumbences in 10% of cases were the clinical signs reported in symptomatic malaria cases. Anaemia and thrombocytopaenia made up the majority of the haematological abnormalities observed in malaria-infected buffaloes. This is the first report about *Plasmodium bubalis* in Iraqi buffaloes.

Key words: apicomplexan, buffalo, Iraq, malaria, *Plasmodium* spp.

INTRODUCTION

Buffaloes in Iraq are mostly dairy animals, and never put to work. They are the major source of thick butter fat (gaymar) and can produce high amounts of milk as well as meat conversion despite low-quality forage (Juma, 1997; ALsaedy, 2007). Some time ago, buffaloes have been neglected in Iraq for some reasons, including military conflicts and protozoan infections such as *Babesia*, *Theileria*, and *Trypanosoma*, which significantly affected the economic viability of these animals.

Apicomplexan parasites of the genus *Plasmodium* are a well-known causative agent of malaria in humans and animals. They are transmitted by bites of female *Anopheles* mosquitoes. Malaria parasites (*Plasmodium* spp.) can be responsible for infecting several vertebrate hosts, such as ungulates (hoofed mammals). Malaria parasite *Plasmodium bubalis* has been first described by Sheather (1919) through microscopic observation of blood smear from water buffaloes in India. The species of *Plasmodium bubalis*, described in water buffaloes, became distributed worldwide (Rao, 1938; Riaz-ul-Hassan, 1953; Shastri *et al*., 1985; Kolte *et al*., 2002; Sundar *et al*., 2004; Shinde *et al*., 2005). Recently, *Plasmodium bubalis* has been molecularly characterised by Templeton *et al*. (2016 a,b), who isolated two separate *Plasmodium* sequences from water buffaloes in Thailand and Vietnam, which, in the initial stage were known as *Plasmodium bubalis* types I and II. Besides, the first case of water buffalo malaria parasites *(Plasmodium bubalis)* in PCR-based surveillance was documented in Nepal by Kandela *et al*. (2016). Currently, there are no data about malaria infection in Iraqi buffalo. Mosul city is the provincial capital of Nineveh, and one of the richest Iraqi governorates with thousands of buffaloes due to the nature and water resources from Tigris and Greater Zab rivers which irrigate much of Mosul.

In the present study, microscopic examination of blood smears was performed to detect malaria (*Plasmodium* spp.) as a new apicomplexan parasite in Iraqi buffalo, and subsequently by nested PCR assays to confirm the infection. The clinical and haematological parameters of buffalo malaria cases presented to a hospital were also recorded in this study.

MATERIALS AND METHODS

Ethical approval

The study has been approved by the Department of Internal and Preventive Medicine, College of Veterinary Medicine, University of Mosul, Mosul, Iraq.

Study area

This research was conducted in the Veterinary Teaching Hospital, College of Veterinary Medicine, University of Mosul (VTH-UoM), Mosul city, Iraq, between July 2019 and January 2020.

Samples and data collection

A total of 70 cases of domesticated water buffaloes (*Bubalis bubalis*) of different ages and both sexes from privately-owned farms in Mosul city (Mosul forest Al-Kabat near the 5th bridge, Al-Rashydia, Hawi Al-kanesa, and surrounding regions (Badosh, Al-Salamia, Al-Shalalat, Yarmjah) on the banks of the Tigris River were referred to the VTH-UoM, Mosul, Iraq. Data on characteristic animals and herds are collected through questionnaires, and complete clinical examinations were done. All the 70 cases were confirmed by microscopic examination of thin and thick blood smears made available on site. Blood samples (5 mL) collected from each animal were positive for *Plasmodium* parasite in the blood smear. As for the negative cases, they were considered as control animals for the comparison of the clinical and haematological changes. Blood was divided into two aliquots. One of them was anti-coagulated with EDTA for haematology profile evaluation, the other was used for DNA extraction. These aliquots were stored at –20 °C until the time of performing the analysis.

Blood smear preparation and microscopic examination

Preparation of all air-dried thick and thin fresh blood smears was made right after the blood was extracted, fixed and then stained with Giemsa and Leishman according to Sathpathi *et al*. (2014). All thick and thin blood films were examined using light microscope at a magnification of ×1000 with immersion oil (BX51, Olympus, Tokyo, Japan).

Haematological studies

The haematological parameters including red blood cell counts (RBC), red cell distribution width (RDW), white blood cell count (WBC), packed cell volume (PCV), haemoglobin (Hb), platelet count and erythrocyte indices: mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC) were analysed by using Abaxis Vet scan HM5CVS2 Hematology Analyzer, eBay com.

DNA extraction and PCR

Blood samples of 11 positive buffaloes during microscopic examination of stained blood smears were chosen to be evaluated by nested PCR test. Genomic DNA was made available from blood samples using a commercial kit (QIAGEN DNeasy Blood and Tissue kits, QIAGEN, Germany), following the manufacturer's instructions, and kept in storage at 4 °C. PCR assessments were made as stated by Martinsen *et al*. (2006). Amplification of the *Plasmodium cytb* gene was performed by nested PCR using *Plasmodium* specific primers DW2 (TAATGCCTAGACGTA TCCTGATTATCCAG) and DW4 (TGT TTGCTTGGGAGCTGTAATCATAATG TG) for round PCR. The primers DW2 and DW4, used in *Plasmodium* cytochrome b gene (*cytb*) gene amplification, were also used in other researches for *Plasmodium* screening from hoofed animals (Boundenga *et al*., 2016; Templeton *et al*., 2016b; Asada *et al*., 2018; dos Santos *et al.,* 2019, Kandela *et al*., 2019). PCR rounds were conducted for 40 cycles with denaturation at 94 °C for 20 s, then subjected to annealing and extension at 62 °C for 3 min (Kandela, *et al*., 2019). Deionised nuclease-free water as the negative control in PCR amplification. The PCR products were analysed by separation on 1.5% agarose gel electrophoresis, then stained by ethidium bromide and photographed.

Statistical analysis

The data analysis was done with SPSS (Version 17; SPSS Inc., Chicago, USA). A value of $P<0.05$ was deemed to be of statistical significance.

RESULTS

Out of 70 blood samples, 17 (24.28 %) were malaria cases. Among the infected buffaloes, 10 (14.28%) were female, 4 (5.7%) were male and 3 (4.2%) were calves, and the remaining 53 (75.71%) were malaria-negative. Of all malaria-infected buffaloes, 6 (35.3%) cases were asymptomatic (occult infection), while 11 cases (64.7%) showed clinical signs of high fever as the first sign, anorexia, reduced rumen motility, increased respiratory and pulse rates (particularly if animals are moved), muscle tremors, emaciation, pale mucous membranes as well as recumbences then death of one infected calf (Table 1).

Following a thorough microscopic observation of Giemsa and Leishman stained blood smears of sick buffaloes, the different developing stages of *Plasmodium* pa-

Table 1. Comparison between clinical parameters in healthy buffaloes and buffaloes with malaria. Data are presented as mean±standard deviation

Clinical parameters	Non-infected buffaloes (n=53)	Infected buffaloes (n=17)
Temperature (°C)	38.18±0.20	40.43±0.15*
Respiration (min^{-1})	18.92±0.76	53.17±2.01*
Pulse (min^{-1})	57.80±1.35	88.40±1.05*
Rumen motility (per two min)	2.26±0.26	1.00±0.01*

Student's t-test: * $P<0.05$.

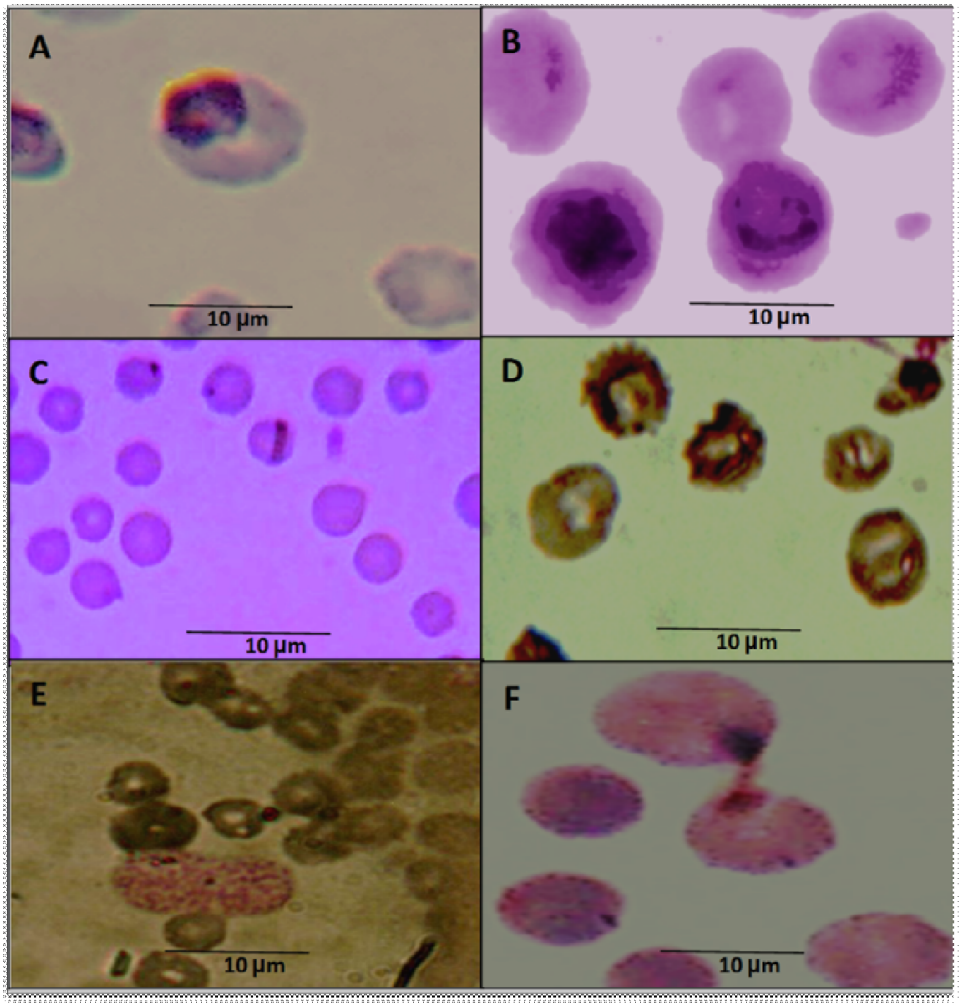

Fig. 1. Microscopic images of *Plasmodium* parasites in water buffaloes blood smears stained with Giemsa (A–C) and Leishman (D–F) stains, under oil immersion at 1000×.

rasites inside erythrocytes were observed with approximately 0.05–0.3% parasitaemia by eye (Fig. 1A-F). The developed amoeboid trophozoites measured 1.3 to 1.7 µm in diameter and were characterised by harbouring brown hemozoin pigments; infected erythrocytes appeared larger, spherical, with hyaline appearance in comparison with the normal erythrocytes (Fig. 1A). Other trophozoites appeared larger, spherical and measured up to 5.0 µm in diameter (Fig. 1B). Band shaped trophozoites like trophozoite of *P. malariae* were also seen in some buffalo blood smears stained with Giemsa reagent (Fig. 1C). Hemazoin crystals were frequently rod-shaped, with two vacuoles (Fig. 1D). The schizonts of *Plasmodium bubalis* elongated to an oval shape were seen firstly in Leishman stained blood smears (Fig. 1E). Also, male and female gametocytes occupying the entire erythrocyte with brown pigments (hemazoin) measuring 6 to 8 µm in diameter (Fig. 1F) were observed.

Haematological parameters of clinically infected buffaloes (n=17) showed a significant decrease in the mean levels of

Table 2. Effects of *Plasmodium* infection on haematology parameters (mean±standard deviation) of buffaloes.

Parameters	Non-infected buffaloes (n=53)	Infected buffaloes (n=17)
RBC ($\times 10^6$/µL)	11.03 ± 1.06	6.50 ± 0.09*
WBC ($\times 10^3$/µL)	14.04 ± 1.01	9.20 ± 0.06*
Packed cell volume (%)	33.03 ± 1.47	19.04 ± 0.19*
Haemoglobin (g/L)	140.30 ± 16.0	60.50 ± 1.30*
RDW (%)	56.32 ± 3.12	22.03 ± 1.03*
Platelets ($\times 10^3$/µL)	195.30 ± 19.50	92.32± 9.10*
MCHC (g/L)	39.10 ± 1.05	32.06 ± 0.11*
MCH (pg)	13.14 ± 1.04	16.02 ± 0.19*
MCV (fL)	361.00 ± 21.5	540.10 ± 1.10*

Student's t-test: * P<0.05.

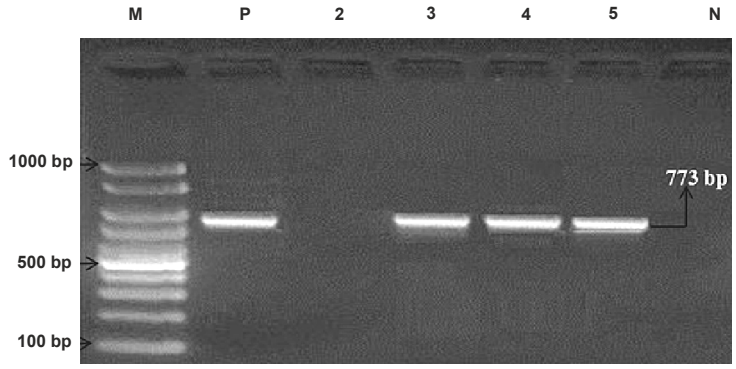

Fig. 2. Agarose gel electrophoresis presenting the size of the amplified products by PCR utilising primers targeting mitochondrial cytochrome b (*cytb*) gene. Lane M: DNA marker (1000 bp); lane P: *Plasmodium* positive control (genomic DNA from *Plasmodium* shows amplification product of ~ 773 bp); lanes 3–5: *Plasmodium* positive samples from domestic buffaloes; lane N: negative control (deionised nuclease-free water).

HCT, Hb, RBCs, RDW value, WBCs, platelets count. All erythrocyte indices MCV and MCHC were also decreased in the samples derived from *Plasmodium*-infected buffaloes in a comparison with the hematological parameters of healthy buffaloes (Table 2).

DNA sequences of three positive PCR products (3, 4 and 5) after amplifying with *Plasmodium*-specific primer were 773 bp long and equal (Fig. 2).

DISCUSSION

In the current study *Plasmodium bubalis* (Malaria) parasites were detected in Iraqi buffaloes from Mosul and caused severe clinical signs with high parasitaemia although an early study on the malaria parasite of buffalo determined that the pathogen caused only mild symptoms and buffaloes were self-cured (Riaz-Ul-Hassan, 1953). Also, the death of some animals, especially among newborn buffalo calves was recorded. There was one mortality case noted by Templeton *et al*. (2016a), but the authors did not determine whether the buffalo (*B. bubalis*) died due to malaria infection or the malaria parasite was an opportunistic infection. Microscopy of the blood smear is still the reference for diagnosing malaria (WHO, 2015). Morphology of the *Plasmodium* parasites was verified in Giemsa- and Leishman-stained blood smears. Even though Giemsa staining is by far the most common practice, the Leishman staining technique offers a superior image of the nuclear chromatin pattern of cells (Sathpathi *et al*., 2014). The existence of hemozoin crystals was indicative of malaria parasites in Iraqi buffaloes, and were often rod-shaped for *Plasmodium bubalis* as mentioned by Sheather (1919) and Templeton *et al*. (2016a).

Anaemia, leukopaenia and thrombocytopenia were the most commonly faced haematological abnormality findings noted in buffalo malaria infection in this research. The pathogenesis of anaemia in malaria has diverse factors and remains inadequately understood; such as mechanical destruction of the parasite-infested red blood cells, decreased RBC levels in the bone marrow, and phagocytosis of parasitised RBC are some of the systems believed to be involved (Jain *&* Kaur, 2005). Leukopaenia was frequently found in total WBC alteration and happened in malaria patients (Rasheed *et al*. 2009). The reason for thrombocytopenia in human malaria is not known; but elevated platelet lysis and decreased platelet lifespan during malaria are believed to take place by both non-immunological destructions and also immune systems that involve particular platelet-related IgG antibodies binding to the malarial antigen in the platelets, which is frequently linked to palpable splenomegaly and the circulation of immune complexes (Prajapati *et al*., 2018). The platelet level declines when the malaria parasitaemia rises, and thrombocytopenia may be utilised to verify the existence and seriousness of malaria (George *&* Ewelike-Ezeani, 2011). It is possible to use thrombocytopenia to support the diagnosis of malaria, besides the medical indices in situations where diagnosing with the microscope is insufficient, as when there is low parasite density (Awoke *&* Arota, 2019). Moreover, negative results obtained in PCR study of 9 samples from eleven cases that were positive for *Plasmodium* parasite in blood smears could be dependent on the low parasitaemia when the blood was extracted. It is known that low parasitaemia was earlier mentioned in infected hoofed animals (Templeton *et al*., 2016b; dos

Santos *et al.,* 2018). The life cycle of *Plasmodium* in vertebrate hosts could have a long-lived dormant presence in the liver, with the parasite sequestered from the normal circulation, resulting in extremely low parasitaemia with the lack of an immunosuppressed state, as earlier stated in water buffaloes (*Bubalus bubalis*) (Sheather, 1919).

This is the first report on *Plasmodium bubalis* in domestic water buffaloes in Iraq. Farmers and veterinarians are ignorant of malaria infection in buffaloes and as a result no precautions and interventions have been initiated from both government and relevant government agencies. We also concluded that *Plasmodium bubalis* can cause significant haematological changes with high frequency of thrombocytopaenia, leukopaenia and anaemia. The blood changes were so characteristic that the diagnosis of buffalo malaria should always be considered in the presence of above findings.

CONCLUSION

Plasmodium bubalis was detected for the first time from the domesticated water buffalo of Mosul, Iraq by microscopy and PCR assay. Clinical and haematological abnormalities were found out during infection.

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