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***Corresponding Author**
Khawar Ali Shahzad
E-mail
khawar.ali@iub.edu.pk
khawar7bar@yahoo.com

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Prevalence and identification of zoonotic haemoparasites on bullfrogs (*Hoplobatrachus tigerinus*) as potential vectors for diseases in humans

Areeba Fatima[1], Ayesha Mushtaq[1], Muhammad Rafiq[1], Aiman Saeed[1], Muhammad Rizwan[1], Zia Ur Rehman[2], Mushtaq Hussain Lashari[1], Khalid Javed Iqbal[1], Khawar Ali Shahzad[1*]

[1]Department of Zoology, The Islamia University of Bahawalpur, Bahawalpur 63100, Pakistan
[2]Department of Physiology, The Islamia University of Bahawalpur, Bahawalpur, 63100, Pakistan

Abstract

Frogs are an essential part of our ecosystem, but they are affecting humans through zoonotic diseases, and their numbers are also declining all over the planet. The primary reasons are natural changes and microorganisms. We aimed to find the possible cause of their decline and the diseases they spread in the surrounding environment. Haemoparasites can also be a significant cause of diseases in frogs, which later on become vectors for diseases in humans. Among 34 frogs, 15 were infected with *Lankestrella* sp. and *Lieshmania* sp., while 19 were uninfected. Males have a higher rate of infection than females, with a ratio of 8:7, while mature frogs were more infected than immature. complete blood count (CBC) reports showed that the infected frogs were anaemic. The findings of this study provide valuable insights into the health status of bullfrogs in the Cholistan Desert and contribute to the understanding of the dynamics of hemoparasite infections in humans. This information is crucial for developing appropriate conservation strategies, and management plans to protect the bullfrogs from declining and spreading diseases as well as their habitat in the face of increasing environmental challenges. Additionally, the study underscores the importance of continued monitoring and surveillance of hemoparasites in amphibian populations from both ecological and public health perspectives.

Introduction

About 300 million years ago, amphibians were the principal vertebrates to invade the land [1], and about 6200 species of living amphibians are present today [2]. During freshwater cycles, their susceptibility to chemicals makes them good bioindicators of environmental pollution [3]. Amphibians are declining all over the planet, and the primary reasons are natural changes and microorganisms [4]. The International Union for Conservation of Nature (IUCN)world amphibian assessment revealed a decade ago that one-third of the known species of amphibians had disappeared or gone extinct[5], and the main causes include environmental change, natural surroundings annihilation, and arising diseases[6]. By assessing the health of amphibians, it becomes possible to detect environmental toxins and contaminations[7, 8]. In China, the common species *Hoplobatrachus rugulusa* (Chinese tiger frog) is sold as food [9]. So humans can be infected with fatal illnesses through them as they have an essential role in the food web [10], like Salmonellosis, Tuberculosis, as well as Leishmaniasis disease caused by haemoparasites present in amphibians *[11]*. Amphibians live in the most assorted territories, assuming a significant part in the food webs, adding to the regulation of invertebrates in natural environments and human-centered regions. Moreover, they are hosts of various parasites, giving no indications of infection[2]. As secondary consumers in many food chains, amphibians play an important role in the ecology. Tadpoles perform a significant part in recycling nutrients. They can be herbivorous or omnivorous and prey on both invertebrates and vertebrates. Mature amphibians are among the most efficient biological pest controllers. Invertebrates and vertebrates also predate them. Because of their significance in the ecosystem, the fall or extinction of their population has a substantial influence on other organisms as well [12]. The impacts of parasitism on hosts can differ depending upon environmental variations or changes in natural circumstances, affecting the advancement of specific infections and facilitating the onset of infections beginning from a few microorganisms [13]. Wild creatures are exposed to various organisms, including hemoparasites. The Trypanosoma and hemogregarine group [14], *Hepatozoon* sp. [15], Hemolivia [16], Leishmania [11], and Lankesterella [17], are often announced as parasites in frogs. Parasites of the phylum Apicomplexa, belonging to the order Eucoccidiorida and subclass Coccidiasina, are known as Haemogregarinas. These parasites have a complicated biological cycle that includes hematophagous vectors (ticks, mosquitoes, fleas, and lice) and have been observed to parasitize amphibians, reptiles, mammals, and birds [18]. Lankesterella is a member of the Eucoccidiorida order (subclass Apicomplexa) [19]. It only occurs in vertebrate hosts, where merogony, gametogony, and sporogony occur, and invasive sporozoites grow. Various blood-sucking invertebrates (leeches, mites, and mosquitoes) can act as paratenic vectors, allowing parasite sporozoites to survive [20]. Lankesterella is reported to infect erythrocytes and other cells. Lankesterellidae reproduce asexually in the host's digestive system or throughout the body and are transferred as sporozoites by hematophagous invertebrates such as mites, flies, and leeches [21]. Amphibian health can be accessed through hematologic analysis, which offers insights into physiological processes, disease presence, nutritional status, and the impact of toxins [7]. Erythrocytes, hematocrits, and erythroplastids are blood markers for changes in oxygenation efficiency or oxygenation capacity [22, 23]. Frogs belonging to the family Ranidae are present in Asia and are thought to have spread to the Sahul shelf roughly 10 million years back, where they are transmitted into more than 12 species [24]. Genera *Hoplobatrachus* belongs to the family Ranidae in Pakistan [25]. The largest frog found in Pakistan is the Bull Frog (*Hoplobatrachus tigerinus*). During drought and winter, it undergoes hibernation by burrowing in soil [26]. To identify the importance of zoonotic diseases of amphibians it is necessary to detect the parasites first because there is little knowledge regarding zoonoses in Pakistan. Thus, the present study was conducted to gather data on hematologic parameters and analyze the impact of hemoparasites on these values across bullfrogs.

Materials and Methods

Study area

The samples were collected from Bahawalpur city, and the surrounding of Baghdad-ul-Jadeed Campus, The Islamia University of Bahawalpur, Bahawalpur, Pakistan. The geographical coordinates of Bahawalpur are 29° 23' 44" North and 71° 41'1" East. Bahawalpur District covers an area of 24,830 km^2 [27].

Sample collection

Bullfrogs were captured using net traps and kitchen tongs from January 22, 2023, to April 25, 2023, with the average amount of rain during the rainy season. Multiple net traps were strategically placed within the canals and checked every two hours to ensure safe and efficient capture. A total of 34 samples of bullfrogs were collected, including 12 females and 22 males. The frogs were categorized into two distinct age groups: immature and mature. Immaturity was defined by the presence of forelimbs and hind limbs, with either no tail or a tail stub and non-breeding size. On the other hand, mature frogs were identified as those of breeding size. Using the color of the throat, we could determine the gender; males have a darker or green throat while females have a whiter throat. An electronic balance was utilized to measure the frogs' weight (**Table 1**).

Blood sampling

Blood samples were collected by removing the tip of one toe from each frog and by clipping the finger tips, the blood was collected using a heparinized insulin syringe, ensuring minimal invasiveness. After blood collection, the bullfrogs were released alive. The samples were then separated into Ethylenediaminetetraacetic acid (EDTA) tubes for complete blood count (CBC) analysis and Serum separator tubes (SSTs) for additional measurements, such as lipid profile, carbohydrates, and proteins.

Table 1: Total number of samples in various groups.

Gender	Female	12
	Male	22
Developmental stage	Mature	21
	Immature	13
Weight	0g-50g	5
	51g-100g	17
	above 100g	12

Hematological examination

Preparation of blood smear: To prepare blood smears, a drop of blood was carefully placed on a slide using a micropipette, followed by the addition of a second slide. Its edge was placed on the blood drop until the drop spread completely, and then the slide was dragged at an angle of 45º to form a thin blood smear. *Examination of blood smear:* To examine blood smears of frogs under a microscope, the slides were air-dried and then immersed in absolute methanol for 3 minutes to fix them. Then Giemsa stain was utilized for staining. Following staining, the slides were air-dried at room temperature in preparation for microscopic analysis. The examination was conducted using a digital microscope in the Parasitology Lab at the Physiology Department of The Islamia University Bahawalpur, Pakistan, with images viewed on a laptop using Pixel Pro 3.0 software. Lankestrella and Leishmania sp. were identified based on their morphology by comparing them with the results of Gericota, Garner [28] and *Hassan, Saeed [11]*. The hematological parameters assessed were hemoglobin (Hb) concentration, red blood cell count (RBC), and packed cell volume (PCV).

Measurement of Packed cell volume: The PCV of the collected samples was performed manually. It was determined using microtubes centrifuged at 1512 relative centrifugal force (G-force) for 10 minutes using a Hematocrit Centrifuge HC-702. Then, the hematocrit level was assessed using the standardized microtube scale.

Red blood cell count: RBCs were counted using a haemocytometer (Precicolor, HBG, Germany). The solution for RBCs was prepared, and cells were counted by placing the solution in Neubauer's chamber one after the other. Heyem's saline was used as an RBC count solution. A diluting solution was used to dilute the solution at a ratio of 1:200. In a red bead pipette, blood was filled up to the 0.5 mark. The solution was then drawn up to mark 101 on the RBC pipette. The blood was well mixed in the pipette. The first few drops of solution were discarded, and the solution was filled into Neubauer's chamber. It was ensured that the chamber was free of air bubbles and that the cells were distributed equally across the defined area. The solution had to be set within two minutes. The total number of cells in Neubauer's chamber was counted at 40X. One central secondary square and four corner secondary squares containing 25 tertiary squares were counted and the sum of the cells in five secondary squares. After calculating the cells, the formula was used to calculate the number of RBCs.

$$RBCs = Average\ count \times 200 \times 25 \times 10$$

Where
200 = Dilution Factor
25 = Area Multiplication Factor
10 = Depth of Chamber

Hemoglobinlevel: For the determination of haemoglobin level, N/10 HCl up to its lowest mark was filled in a hemoglobinometer tube with the help

of a dropper. Using Sahli's pipette, blood was taken up to 20µL and delivered to N/10 HCl in the hemoglobin tube. Hemoglobin to hematin conversion is completed after 10 minutes of mixing the solution. To match the colour of the solution with the colour of the standard glass of the comparator, distilled water was added drop by drop. Readings were noted, which provides the hemoglobin concentration in 100 ml of blood.

Statistical analysis

The prevalence of haemoparasites was calculated using the below given formula:

$$\text{Prevalence } = \frac{The\ Number\ of\ frog\ infected}{Number\ of\ frogs\ examined} \times 100$$

Data analysis was done by the software SPSS 25.0. Data from the sampled locality in Southern Punjab was used to investigate the relationship between host factors such as gender, weight, age, and parasite infection. The prevalence of infection was investigated using three-way ANOVA, as was the comparison of CBC across age groups. The chi-square statistic was implemented for comparing categorized variables.

Results

A total of 34 bullfrogs (*Hoplobatrachus tigerinus*), 22 males and 12 females, were collected from January through April 2023. The overall average weight of bullfrogs was 83.64g (range 29.77g – 205.91g). 21 frogs were mature and 13 were immature. There was a significant difference in their weight and age. 15 (44.11%) (8 males and 7 females) out of 34 frogs were infected with the blood parasite Lankestrella and Leishmania sp., whereas 19 (55.88%) were uninfected (Fig. 2, Fig. 3, and Fig4). No nematodes were recovered. Infection of *H. tigerinus* in relation to gender (Table 1) showed that females (58%) were more infected with the *Lankestrella* sp. than males (36%). The mean value of Hb, PCV, and RBC × 10^5 of females was 4.7 ± 2.6, 12 ± 7, and 3.63 ± 3.48, respectively, which is smaller than the mean value of males, which is 9.0 ± 4.1, 21 ± 12, and 8.63 ± 9.23, respectively. The relation of *H. tigerinus* parasite infection with age group showed that mature frogs (52%) were more infected than immature frogs (31%) with *Lankestrella* sp. There was a significant difference in the infection of the mature group compared with the immature. The mean values of RBC, Hb, and PCV were $6.55\times10^5 \pm 6.36\times10^5$, 7.3 ± 3.3, and 18 ± 12 while in immature the mean values were $7.37\times10^5 \pm 10.41\times10^5$, 7.6 ± 5.3, and 17 ± 10.

The prevalence of infection increased with the decreased weight of the host. There are three groups of weight; Group 1 contains 0-50gm of frogs, Group 2 contains 51-100gm of frogs, and Group 3 contains frogs above 100gm. Group 1 has an infection rate of 60%, while group 2 has 35% and group 3 has 50% frogs infected.

The RBC, Hb, and PCV counts for Group 1 were 2.86 ± 4.10, 4.4 ± 2.6, and 9 ± 7 for Group 2 these were 8.09 ± 9.75, 8.4 ± 5.0, and 20 ± 11, while for Group 3 these were 6.79 ± 6.19, 7.3 ± 2.4, and 18 ± 11 respectively. The RBC count, Hb, and PCV in infected hosts were lower than in uninfected hosts. The RBC, Hb, and PCV values in infected hosts were 5.44 ± 6.18, 5.1 ± 2.1, and 12 ± 11, respectively. In uninfected hosts, the RBC, Hb, and PCV values were 7.99 ± 9.21, 9.3 ± 4.4, and 22 ± 10, respectively.

Discussion

Mammals, birds, reptiles, crocodiles, and amphibians are the vertebrate hosts of haemoparasites [29]. According to Žičkus [30], frogs have been found to be infected by species of Haemogregarina, Hepatozoon, Lankesterella, and Schellackia. Leal, O'dwyer [31] researched in Brazil on anurans of three families, their samples were positive for Haemogregarines and Trypanosoma sp. with prevalence rates of 10% and 20%, respectively. In the present study, the prevalence is 44%, and the anurans were infected with the Haemogregarine species Lankestrella, and no Trypanosoma sp. was recovered. Paperna and Martin [32] studied the bullfrogs of Nigeria, and they were found to be infected with Lankestrella at the gametogenic stages. similarly, this study also reveals the presence of Lankestrella in bullfrogs of Pakistan at the gametogenic stage. Recently in a study by the Netherlands, Cook [33] on an African frog 29 species, microfilaria nematode species were observed. However, in our study, nematodes were not detected. To the best of our knowledge, these parasites have not been found in the amphibians of Pakistan. *Hassan, Saeed* [11] studied Leishmania in *Mabuya sp.* Lizards and *Buforegularis* toads of Sudan and according to their findings, lizards are infected with this haemoparasite, but the present study searches Leishmania in frogs as frogs also prey upon small lizards. Therefore, this parasite can also infect amphibians, and then into humans. However, the

Table 2: CBC count of samples according to gender, age, weight, and infectious frogs.

		RBC $\times 10^5$			Hb			PCV			Parasite	
		Mean	SD	Variance	Mean	SD	Variance	Mean	SD	Variance	Yes Count	No Count
Gender	Male	8.63	9.23	85.14	9.0	4.1	16.4	21	12	145	8	14
	Female	3.63	3.48	12.13	4.7	2.6	6.6	12	7	50	7	5
Age	Mature	6.55	6.36	40.50	7.3	3.3	10.7	18	12	145	11	10
	Immature	7.37	10.41	108.34	7.6	5.3	8.7	17	10	110	4	9
Weight	0-50	2.86	4.10	16.78	4.4	2.6	6.7	9	7	49	3	2
	51-100	8.09	9.75	95.11	8.4	5.0	25.4	20	11	132	6	11
	above 100	6.79	6.19	38.26	7.3	2.4	5.7	18	11	130	6	6
Parasite	Yes	5.44	6.18	38.15	5.1	2.1	4.5	12	11	121		
	No	7.99	9.21	84.83	9.3	4.4	19.3	22	10	95		

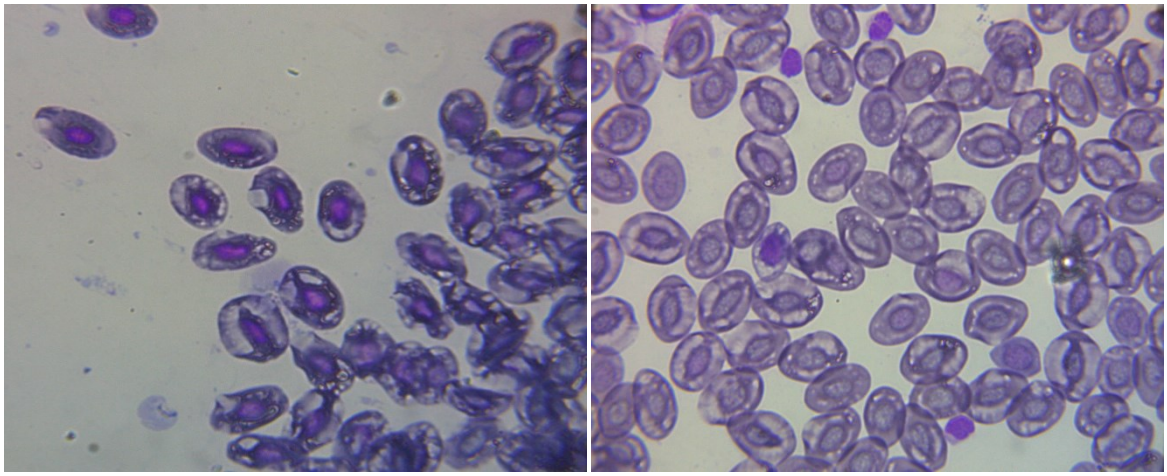

Fig. 2: Presence of *Lankestrella sp*. in Erythrocytes of *Hoplobatrachus tigerinus*

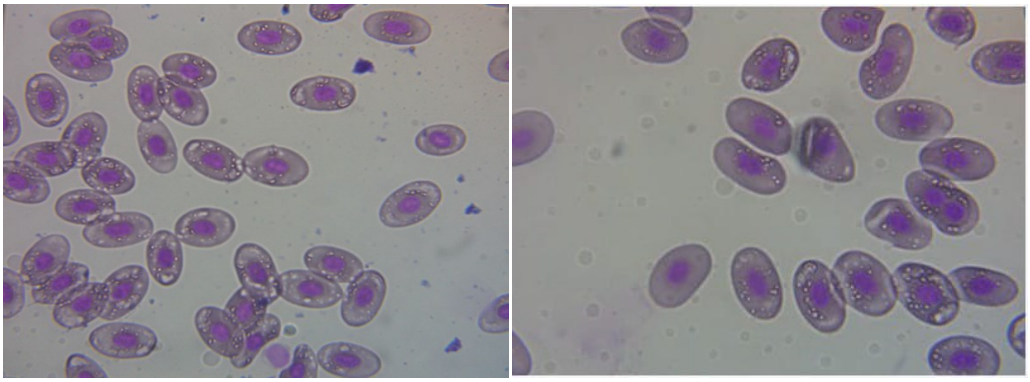

Fig. 3: Presence of *Lankestrella sp.* Gamonts in erythrocytes of *Hoplobatrachus tigerinus*

study conducted by [34], concluded that hemoparasites did not affect amphibians in Nigeria and no difference in the haematological parameters of infected and non-infected frogs. In our study, the infected frogs had decreased RBC Count, PCV and Hb, which resulted in anaemia due to the infection, this differentiation of results may be due to the difference in environment of both countries. Mohamed [12] studied the effects of haemoparasites on toads in relation to their weight, age and gender, there results correlates with the present study.

This study is the first to investigate haemoparasites in amphibians of Pakistan, and it serves as an initial step in a subsequent phylogenetic and taxonomic characterization of Pakistan's amphibian haemoparasites. Morphological methods were employed to conduct the examination. Future research should encompass molecular and morphological analysis, with the potential inclusion of definitive hosts or vectors, such as mosquitoes and leeches. This study contributes to a broader understanding and awareness to safeguard human health from these

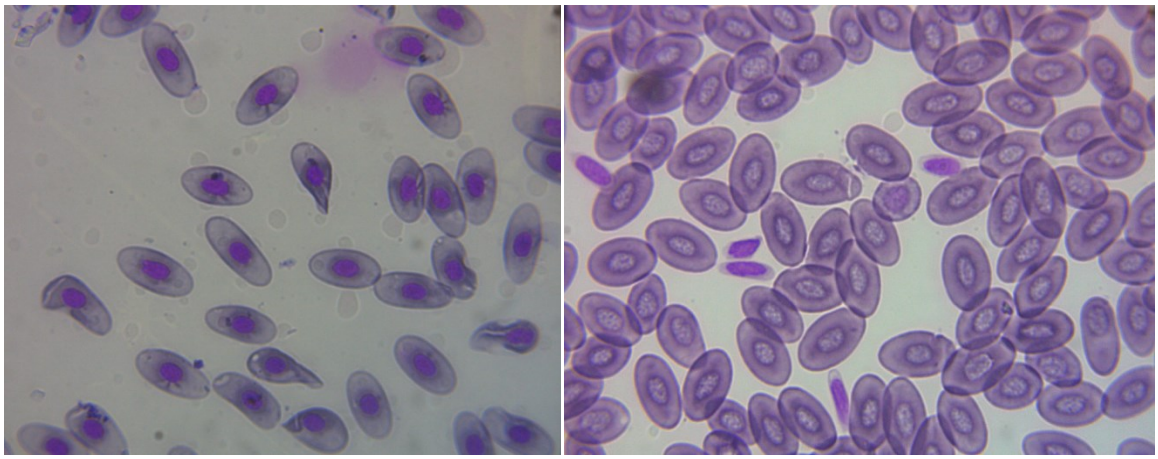

Fig. 4: Presence of *Leishmania sp*. in *Hoplobatrachus tigerinus*

emerging threats by highlighting the zoonotic diseases. It emphasizes the importance of hygiene practices and preventive measures as in some countries frogs are used as food.

Conclusion

It is concluded that infected males are 8/22 while infected females are 7/12 whereas, the overall infection rate was 44.11%. Age also impacts the prevalence of parasites, as infection rates increase with age, and hosts with increased weights are more anaemic and susceptible to infections than lower-weight hosts. In accordance with the localities, the prevalence was different among both localities. This may be due to the climate difference between these areas. Humans can also be affected by these infected frogs and can contract diseases like Leishmaniasis from them. It is imperative to take preventive measures and maintain hygienic conditions in order to prevent humans from such zoonotic diseases.

Conflict of interest

The authors declare no conflict of interest.

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