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A preliminary study of haemoparasites in marsh frogs, *Pelophylax ridibundus* (Ranidae) from Iran

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Abstract
Anurans are known to harbor different type of intra- and extracellular blood parasites. Apicomplexan parasites, order Coccidea as well as Kinetoplastea are the most commonly reported blood parasites from frogs. The present study was conducted to survey haemogregarines and trypanosomes, two groups of haemoparasites in marsh frog (*Pelophylax ridibundus*) from north of Iran. The frogs were captured and blood samples were obtained by insulin syringe. Blood smears stained by gimsa and were examined for the presence of haemoparasites. The microscopic diagnosis detected *Hepatozoon magna* as the only haemogregarines. Six morphological forms of *Trypanosoma* sp were observed as described in the literature, with slender to oval and broad forms. This is the first report on blood parasites of *Pelophylax ridibundus* in Iran.

Keywords: Haemoparasite, *Hepatozoon magna*, *Trypanosoma*, Frog, Iran

1. Introduction
Amphibians are exposed to a wide variety of intracellular and extracellular parasites ranging from protozoans to extracellular nematode microfilariae as well as uncertain identity of intracellular parasites such as the viral and bacterial infections [1-5]. Haemogregarines are comprised of a large group of diverse heteroxenous adeleid coccidian parasites which have exploited in a wide group of tetrapod vertebrates including reptiles, amphibians, fishes and their haematophagous invertebrates [1, 6]. Haemogregarines are investigated in three families namely the Haemogregarinidae Léger, 1911, Hepatozoidae Wenyon, 1926, and Karyolysidae Wenyon, 1926.
The haemogregarines have generally the life cycle comprises roughly four stages: merogony and gamogony in the vertebrate host, and fertilization and sporogony in the invertebrate host. Gamonts are usually intraerythrocytes and observe variable in shape; some present a broad shape, others have a narrow and elongate shape. The main genera of this group are the *Hepatozoon* (Family: Hepatozoidae) that they are transmitted by a variety of arthropods, specially mosquito [1, 7]. Prior the amphibian haemogregarines were placed in the genus *Haemogregarina* Danilewsky, 1885 [8]. However Smith [6] basis of sporogonic development, transferred most of haemogregarines into the genus Hepatozoon Miller, 1908 (Adeleorina: Hepatozoidae) [6].
Haemoparasites of the genus *Trypanosoma* belonging to kinetoplastida order, frequently were reported from anurans species. Podlipaev [9] reported following species of trypanosomes from frogs: *T. inopinatum* Sergent et Sergent, 1994 (Edible Frog *Rana esculenta* and Common Frog), *T. elegans* Franca and Athias, 1906 (Edible Frog), *T. rotatorium* Mayer, 1843 (Edible Frog, Common Frog, Marsh Frog and Common Toad), *T. hylae* Franca, 1908 (Common Tree frog *Hyla arborea*), *T. costatum* Mayer,1843 (Edible Frog), *T. ranarum* Danilewsky, 1885 (Edible Frog). The taxonomy of anurans *Trypanosoma*, due to the great polymorphism in this genus is confusing and numerous species of same parasites have been described in different names [10, 11]. According to Bardsley and Harmsen [12], all highly polymorphic trypanosomes in the world wide anurans that transmitted by leeches, should be included to the *Trypanosoma rotatorium* complex [13]. They also noted these trypanosomes should identify basis of restriction to a particular species of a vertebrate host, instead of only morphologically.
Pelophylax ridibundus (marsh frog) lives in mixed and deciduous forests, forest steppe, semidesert and desert zones is a semiaquatic species that inhabits a wide variety of flowing and stagnant water habitats, from shallow puddles and ponds to large lakes and rivers, as well as mountain streams.

The species that originally described from Kazakhstan [14] distributed in Central Europe, northwards to the Baltic Sea and southwards to the Mediterranean regions, eastwards to Asiatic Russia, and southwards to the Middle East [15]. In spite of wide distribution of anurans in Iran, there is still no information on blood parasites. The purpose of this study was to investigate and identify of blood parasites of *P. ridibundus* (a species with wide distribution in Iran) occurring on the Guilan, Iran.

2. Materials and Methods

Frogs of *P. ridibundus* (n= 10) were collected by net in Anzali wetland (37°28′ N, 49°25′ W) during spring 2017. Blood was taken from the facial vein, using a 1 ml fixed needle insulin syringe. Thin blood smears were prepared, air-dried, fixed in absolute methanol and stained with Giemsa for 15–20 min [16]. The parasites were investigated by the light microscope under 1000×. Infection intensity of trypanosomes was estimated by calculating the number of parasites per 100 fields of the microscope under 400×. The intensity of intracellular parasites was estimated for 10^4 red blood cells (RBC). All measurements and photographs of fixed specimens were taken using TSVIEW software (version 6.2.4.5). The morphological characteristics of the parasites were evaluated according to Telford [17]. All measurements were given in µm; additionally, LW value (length × width) and the shape index as L/W ratio were calculated. Single factor analysis of variance (ANOVA) was used to analysis of data.

3. Results

On the basis of the morphology of gametocytes, we confirmed the presence of *Hepatozoon magna* and *Trypanosoma sp* in blood smear of *P. ridibundus* (Fig.1 and Fig. 2). A total of 10 individuals of *P. ridibundus* that were investigated in this study, *H. magna* detected just in one sample and it was coexist with *Trypanosoma sp* simultaneously. However, we detected *Trypanosoma sp* in three samples. The mean intensity of *H. magna* and *Trypanosoma* sp were 2.8% and 5% respectively.

Mature *H. magna* found intraerythrocytically. The Measures were 15.96–20.29× 4.12-6.94 µm, LW 65.75–140.81µm, L/W 3.87–2.92 (n=25). The measure of the nucleus were 4.68-6.24×3.43-4.83 µm, 16.05-30.13, 1.36-1.29 µm (Table 1). The shape was observed to lie elongate with broader rounded in anterior end and narrower posteriorly, reflexed and folded upon themselves and occupies a maximum area in the host erythrocytes. The parasites were placed either with the concave border of the host cell nucleus and sometimes remain at one pole of the host cell. The host cell nucleus was displaced laterally or to one pole and frequently appeared lobbed. Hypertrophy and double infection have been seen in infected erythrocytes (Fig. 1).

Different form of trypanosomes were observed from our samples. Six morphological forms of trypanosome are shown in Fig. 2. They were cylindrical rather narrow and the body tapers to a narrow point at each end, broad or round and lacks a free flagellum. They commonly have a conspicuous undulating membrane. The nucleus was oval and elongate. Morphometric data from the *Trypanosoma* species has showed in Table 2.

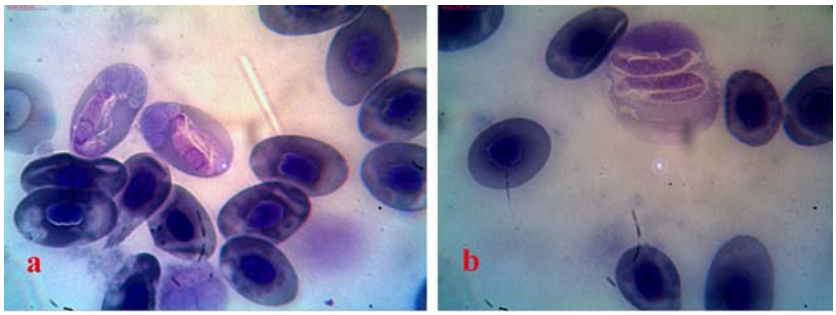

Fig 1: Photomicrographs of *Hepatozoon magna* observed from *Pelophylax ridibundus*

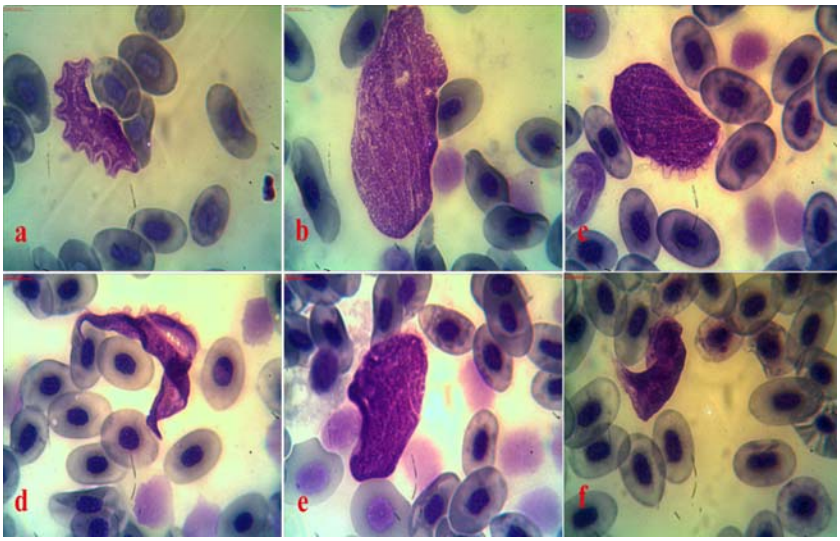

Fig 2: Photomicrographs of six different forms of *Trypanosoma* sp (a-f) from *Pelophylax ridibundus*

Table 1: Morphometric parameters of *Hepatozoon magna* in different studies

Parasite and reference	Gametocyte		Nucleus	
	Length	Width	Length	Width
H. magna Grassi and Feletti, 1891, *Pelophylax esculentus*, Europe	18	6	4	4
H. magna Ray and Choudhury 1984 [20], *Rana tigrina*, India	12.4± 1.97	4.06± 0.23	4.4± 0.67	3.3± 0.28
H. magna (from the present study)	18.03±1.59	4.92± 0.6	5.52± 0.45	4.03± 0.46

Table 2: Morphometric parameters of different forms of *Trypanosoma* sp. from *Pelophylax ridibundus*

Form	Length				Width			
	N	LIM	X	SD	N	LIM	X	SD
I	10	33.72-39.71	36.72	4.18	10	10.54-13.44	11.99	2.02
II	9	43.56-55.73	49.65	7.91	9	25.78-30.10	27.94	2.8
III	12	33.58-38.58	36.08	3.93	12	20.09-23.97	22.03	3.05
IV	9	55.38-62.60	58.95	4.69	9	13.72-16.09	14.91	1.53
V	7	40.57-48.95	44.76	4.52	7	23.45-30.49	26.97	3.8
VI	10	27.33-31.93	29.63	3.21	10	12.53-16.09	14.31	2.48

4. Discussion

Haemoparasites of the genus *Hepatozoon* have been described in a range of vertebrates host including mammals, birds, reptiles, crocodilians and amphibians [6]. According to Zickus [13] species of *Haemogregarina*, *Hepatozoon*, *Lankesterella*, and *Schellackia* were recorded in frogs. Leal *et al.* [11] with study on three families of anurans in Brazil found 10% of samples for haemogregarines and 20% for *Trypanosoma* sp were positive. Recntly in a study by Netherlands *et al.* [18] on biodiversity of haemoparasites in 29 species of African frogs, five groups of haemoparasites including *Hepatozoon*, *Dactylosoma*, intraerythrocytic organisms of a viral or bacterial nature, species of the genus *Trypanosoma* and microfilarid nematode species were observed.

In the present study, *Hepatozoon magna* and *Trypanosoma sp* for the first time reported of *Pelophylax ridibundus* from Iran. The prevalence of *H. magna* (10%) observed in this study is similar with results of Leal *et al.* [11]. In contrast, the prevalence of *Trypanosoma* sp in our study (30%) was higher. However, it is not possible to compare of the results of this two studies, because of difference in the number of samples and species diversity. Desser *et al.* [19] in his study on three species of anurans reported 20% of samples by trypanosomes and 42% of samples by *Hepatozoon* and *Lankesterella* were infected. Morphological measurements of the *H. magna* in this study were completely different from previously described of this species from *Rana tigrina* in India [20]. *H. magna* measured in this study were bigger in size of parasites and nucleus. However the present species seems to proximate with *H. magna* in *P. esculentu* from Europe described by Grassi and Feletti, 1891. It could be explain due to strong familial specificity among the Haemogregarinidae species as regards their host and it can be lead to more morphological similarity in same hosts [21]. This host specificity may be use as important guide to the identification of haemogregarines in same families. This information contention confirmed both under natural as well as under experimental condition [20]. Some morphological difference in gametocytes in various species of hosts may be referred to unknown part of the developmental cycle of the parasites [20].

The nucleus of infected erythrocytes sometimes undergoes considerable change in their shape, size and structure [20]. In our study, infected cells were frequently hypertrophied and host cell's nucleus were sometimes lobular and becomes more dense at the one corner (Fig.1). Mohammed and Mansour [21] reported wrinkled, collapsed and empty sac corpuscles in infected toads by haemogregarines. Similar results were observed in *H. boueti* and *H. andamanensis*, which either fragment or cleave the host cell nucleus [20].

Trypanosomes are the common species of haemoparasites in frogs and transmission is known to occur due to haemophagues of leeches. However some researcher considered dipeterans as a possible vector of anuran [22]. *T. rotatorium* frequently recorded from *P. redibundus*. It identified by very large, broad trypanosomes without free flagella. However in some resource have reported medium sized, to very large round, oval or irregularly shaped with either no flagellum or only a small internal remnant [23].

We recorded six morph of *Trypanosoma* sp in this study (Fig. 2 and Table 2). The morphological analysis of *Trypanosoma* species recorded in present study in most forms are similar to made by Zickus [13] and Lemos *et al.* [24] from different form of *T. rotatorium*. Occurring of polymorphism in *T. rotatorium* have been reported by many researcher [19, 25]. Lemos *et al.* [24] recorded four morphotype of *T. rotatorium* from *Leptodactylus fuscus*. Scorza and Dagert [26] suggested all trypanosomes in anurans in the Americas should be recognized as variations of *T. rotatorium*. However, we recorded different type of trypanosomes in this study. We have trouble in identification due to polymorphic variability reported by different researcher. Miyata [27] described some characters of these parasites such as position of kinetoplast, nucleus and free flagellum. However, most samples were difficult to determine of this characters or sometimes no visible.

The present description of *H. magna* and *Trypanosoma sp*, using morphological methods is the first study of haemogregarines and trypanosomes in anurans of Iran and thus represent an initial step in the future taxonomic and phylogenetic characterization of Iranian amphibian haemoparasites. Future studied should include both morphological and molecular identification along with of possible definitive hosts or vectors such as leeches and mosquitoes.

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