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Molecular detection of *Hepatozoon* in the blood of snakes from Mizoram, India

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Abstract
The main objective of this study is to record the prevalence of haemoprotozoan *Hepatozoon* in various species of snake blood by conventional blood film examination as well as by molecular technique. A total of 100 blood samples from both poisonous and non-poisonous snakes were examined over a period of one year from July, 2016 to June, 2017 from different parts of Mizoram. Twenty blood samples were found positive for *Hepatozoon* spp. by blood film examination, while twenty five samples were found positive for PCR. Gametocytes of the parasite were observed inside the RBC. Some were found free in the plasma. They were broadly elongate in shape. Nuclei of the infected erythrocytes were pushed to one side of the host cell. Polymerase chain reaction (PCR) selectively amplified a fragment length of about 253bp. The study indicates that *Hepatozoon* infection is quite common in snakes.

Keywords: Snakes, *Hepatozoon*, PCR, Aizawl, Mizoram, India

1. Introduction
Hepatozoon spp. are the most common, widely distributed intracellular haemoprotozoan affecting mammals, amphibians and reptiles inducing snakes[1]. *Hepatozoon* spp. are often found in reptiles, appearing as large banana-shaped gametocytes in the cytoplasm of host erythrocytes the life cycle of this parasite is indirect, with sexual reproduction and schizogony taking place in an arthropod vector. Transmission to vertebrate hosts take place when such infected vector is ingested by a vertebrate host. After ingestion, sporozoites are released and migrate to the visceral organs, mainly to the liver where they undergo merogony. Finally, the merozoites are liberated from ruptured cells and enter into the erythrocytes to form gamonts. The pathogenic effects of hepatozoonosis on their hosts vary from trivial consequences to sever effects on host growth rate and reproductive performance [2, 3]. Studies of this parasite are therefore essential to assess if this haemoprotozoan may pose a risk to pet population including snakes [4]. Such assessments are particularly important in North-Eastern hilly region of India like Mizoram, where host endemicity is high leading to increased extinction risks. Franklin (2005) [5] reported that such assessment criteria is extremely crucial in oceanic islands with high host endemicity level and possibly higher host susceptibility [6].
Mizoram is a hilly state situated at the extreme North-eastern corner of India. Most of the hilly regions are covered with forests. To date, no studies have been carried out to ascertain the prevalence of blood parasites in snakes from this region.
In the present study, we systematically assessed for existence of Hepatozoon species in blood of snakes for the for the first time using molecular and blood smear examination method.

2. Materials and Methods
2.1 Collection of blood
Live snakes were captured after taking utmost precautions throughout the seasons from different localities in the study area. The experiment was started from July, 2016 to June, 2017 and the selected districts of Mizoram were Aizawl, Lunglei, Mamit, Saiha, Champai, Lawngtai and Kolasib. Tail vein of each snake was punctured, thin blood slides were made and quickly air dried and the snakes were then released free [7]. A total of 100 snakes belonging to the families Pythonidae, Viperidae, Elapidae and Colubridae were examined. Approximately 250µl of blood from each snake was also collected in a sterile EDTA containing vial for molecular analysis.

2.2 Examination of blood samples

The blood smeared slides were stained with Giemsa stain as per standard protocol with slight modification [7]. Briefly, the smeared slides were fixed in 100% pure methanol for 3-5minutes. Then the fixed smear was stained with 10% Giemsa stain for 45 minutes. After that slides were washed under running tap water, the slides were air dried and finally examined under oil immersion lens (X100 magnification) for detection of haemoprotozoa.

2.3 DNA extraction from blood samples

DNA was extracted from each blood sample using GeneJET Genomic DNA Purification Kit (Thermo Scientific, USA) as per manufacturer's protocol with slight modifications. Briefly 200µl of whole blood was mixed with lysis solution and proteinase K solution and kept at 56°C for 10 min. Then ethanol (96-100%) was added and mixed by vortexing. The lysate was transferred to the DNA purification column and centrifuged. After washing with buffer twice, the elution buffer was added to the column membrane. Finally it was incubated at room temperature for 2 min and the column was centrifuged. The filtrate was collected and kept in -20 °C.

3. Results

Out of the slides examined 20 were found infected with *Hepatozoon* spp and the result is shown in Table no. 1 and Figure 1. Gametocytes of the parasite were observed inside the RBC. Some were found free in the plasma. They were broadly elongate in shape. Nuclei of the infected erythrocytes were pushed to one side of the host cell. Gametocytes stained dark blue, the nucleus was compact, staining dark blue to red. Twenty five of the 100 blood samples gave positive results (Table no. 2). Some samples which were found negative by blood smear examination showed PCR amplification when the specific primers were used.

An approximately 253bp of the ITS2 region of 16s mitochondrial rRNA of *Hepatozoon* spp was amplified from the collected blood samples for molecular confirmation. Genomic DNA was amplified and the amplification of specific PCR product was checked by gel electrophoresis with 1.5 % agarose gel and viewed in UV transilluminator system (Fig. 2). The data in the table 1 and 2 were statistically insignificant.

4. Discussion

Apparently, the prevalence of *Hepatozoon* spp could be done only by blood smear examination and most haemoparasites are identified by this way. However, it fails to detect during light infection and at the time of low parasitaemic stage. This difficulty would be overcome if blood smear examination is supplemented with molecular techniques [8-9]. In the present investigation, 5 samples which were found negative by blood smear examination but showed positive results using PCR. A similar finding was also recorded by previous workers [10]. It is therefore clear that microscopical examination alone is insufficient for diagnosis of haemoprotozoan infections. The sensitivity of the molecular technique depends on level of parasitaemia and the effectiveness of the primers. However, it is worth to maintain that simply not finding *Hepatozoon* spp. in blood smear does not necessarily means that the sample is negative. Important evidence can be evaluated in conjunction with the molecular data.

In positive cases, gametocytes were found inside the RBC. Some gametocytes were also found free in the plasma. A similar observation was also noticed by Sloboda *et al* (2007) [11]. Harris *et al* (2011) [12] sequenced 18S rRNA for a number of hepatozoon species from a variety of endemic hosts. They assessed phylogenetic analysis to determine if hepatozoon species from reptiles are monophyletic. A similar study was also carried out by other workers [8, 9, 13]. Ujvari *et al* (2004)[14] reported that 100 snakes showing positive for Hepatozoon spp. by PCR, only 75% of these showed positive when bloodsamples were examined. In the present study, slight differences between two methodologies were observed. There are many unknown factors which might give false negative blood smear examination. Notably, low parasitaemia is one of the contributory factors. Results obtained from molecular data augments our diagnostic information. Phylogenetic assessments must be considered for further information and speciation of *Hepatozoon* spp.

Table 1: Prevalence of *Hepatozoon* spp of snakes by blood smear examination

Place	No. of snakes examined	No. of snakes positive for *Hepatozoon* spp	Percentage of infection
Aizawl	15	04	26.67
Lunglei	18	04	22.22
Mamit	10	02	20
Saiha	12	02	16.67
Champai	19	03	15.79
Lawngtai	16	03	18.75
Kolasib	10	02	20
Total	100	20	

Table 2: Showing positive reactions for PCR

Place	No. of snakes examined	No. of snakes positive for *Hepatozoon* spp	Percentage of infection
Aizawl	13	03	23.07
Lunglei	16	03	18.75
Mamit	12	03	25
Siaha	18	05	27.78
Champai	12	03	25
Lawngtai	08	02	25
Kolasib	21	06	28.57
Total	100	25	

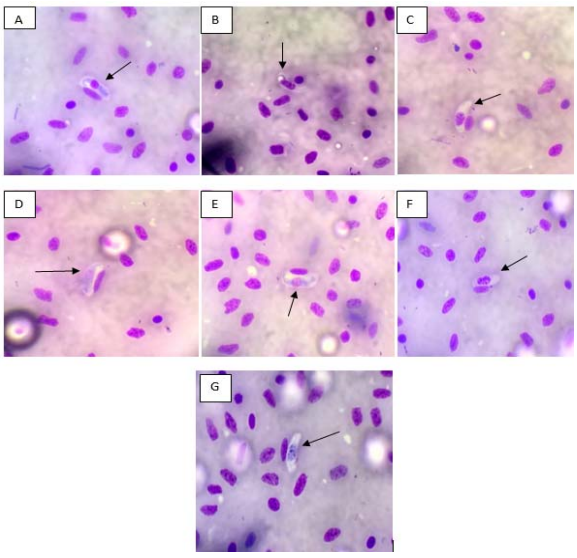

Fig 1: Hemogregarins (*Hepatozoon* parasites) infecting erythrocytes from snakes in Mizoram. Pictures were taken at 100X. (A) Aizawl (B) Kolasib (C) Lunglei (D) Mamit (E) Siaha (F) Champai (G) Lawngtai

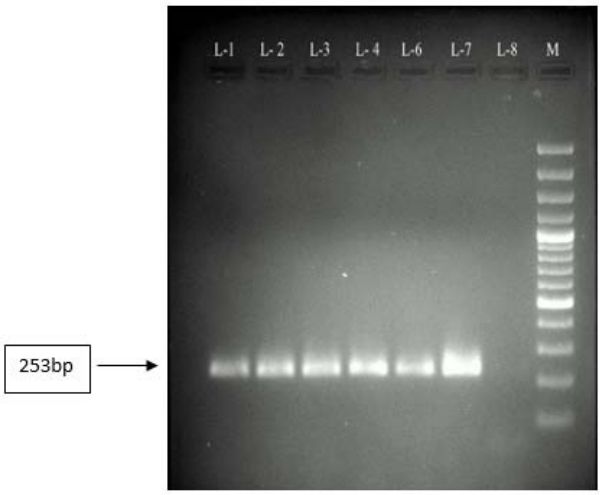

Fig 2: L1 - L6 – Positive amplification of *Hepatozoon* sp.
L 7 – Negative control M – 100bp DNA ladder

5. Conclusion

This is the first report of *Hepatozoon* spp. infecting various species of snakes in Mizoram, India. However, further studies should be carried out to evaluate the ecological relationship of this haemoprotozoan infecting other reptiles in this area

Conflict of interest statement

We declare that we have no conflict of interest.

6. Acknowledgements

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