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Molecular report on blood protozoan *Haemoproteus* sp., from ticks infested ratsnake (*Ptyas mucosa*) in India

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Abstract
A rescued male rat snake was presented with reluctance to move and dullness. Physical examination revealed the presence of ticks over the body surface which was collected for morphological identification. Haematological analysis indicated anemia and leucocytosis in the snake. The ticks were identified as *Amblyomma gervaisi* and *Amblyomma pattoni* based on morphological characteristics. Blood smears revealed the presence of intracytoplasmic halter-shaped gamonts of *Haemoproteus* sp. within the nucleated erythrocytes which was confirmed by Polymerase Chain Reaction (PCR) using genus-specific primers. The snake was treated with Ivermectin and Chloroquine for successive days. These findings contribute to the welfare of snake health and tick-borne diseases, emphasizing the importance of their management and conservation.

Keywords: *Amblyomma gervaisi, Amblyomma pattoni, Haemoproteus* sp., PCR, Rat snake, Tamil Nadu

Introduction
Free ranging and captive reptiles are affected by both ecto-and endo-parasites. Most commonly ectoparasites like flies, fleas, ticks and mites can infest the reptiles and have a detrimental effect on reptile's health and well-being and, if left untreated, can even be a threat to life (Mader, locket). Ectoparasites also known to transmit bacteria, rickettsia, viruses and protozoan parasites to the host, causing discomfort, stress and unease (Miller and Fowler, 2015) [9]. Though numerous parasites cause diseases and mortality in captive reptiles, there are no instances of epizootics associated with parasitism in wild populations. It is essential to comprehend the distribution, propensity for dispersal, and host specificity of the parasites in reptile populations because they can exert influential selective effects on their hosts (Marcus, 1981) [10].

Reptiles are the hosts for wide range of blood parasites. Haemogregarine parasites are usually transmitted by biting midges, louse flies and ticks in reptiles (Zhang *et al.*, 2014) [19]. Although impacts of parasites on reptile health have been hypothesized and pathogenicity has been documented for some species, the haemoparasites of the genus *Haemoproteus* are widely recognized as uncommon and generally less pathogenic vertebrate blood protozoa. But in severe condition, it causes haemolytic anaemia leads to death (Miller and Fowler, 2015) [9].

Materials and Methods
a. **Collection of samples:** A rescued male rat snake was presented to Veterinary Clinical Complex, Veterinary College and Research Institute, Orathanadu, TANUVAS by Forest Department, Thanjavur division, Tamil Nadu with the history of reluctant to move and dullness. The snake was manually restrained and subjected for clinical examination. Physical examination revealed the presence of ticks over the body surface of the snake. A total of 15 ticks were collected in 70% alcohol for morphological identification. The blood sample was collected from the ventral tail vein in blood vacutainer containing heparin as an anticoagulant.
b. **Processing of samples:** The collected ticks were subjected to dehydration procedure in ascending grades of alcohol and cleared with xylene as per Soulsby (1982) [13].

Haematological parameters were analysed based on the standard methods (Coles, 1986) [3]. Thin blood smears were prepared which were fixed with methanol and stained using Giemsa stain. The slides were examined under oil immersion objective of light microscope.

c. **DNA isolation from blood:** DNA was isolated from 200 µl of heparinized snake blood using DNA isolationkit following the manufacturer's instructions (QIAGEN DNeasy ® Blood & tissue kit) and stored at -20 ºC till further use.

d. **Polymerase chain reaction:** The rRNA gene of *Haemoproteus* sp., from the suspected snake blood DNA sample was subjected for Polymerase Chain Reaction using genus-specific primersin a thermal cycler under cyclic conditions with initial denaturation of 94 °C for 5 minutes, denaturation at 94 °C for 30 seconds, annealing at 56 °C for 30 seconds, extension at 72 °C for 30 seconds for 35cycles and final extension at 72 °C for 5 minutes. The PCR amplicons were analysed in 1.5% agarose gel electrophoresis and viewed under a UV transilluminator.

Results

a. **Clinical examination:** The snake was dull and anaemic during clinical examination. The haematological and biochemical analysis of the blood sample revealed the following parameters (Table. 1). The snake's blood picture undoubtedly showed it was anaemic, and its elevated heterophil count indicated the presence of an infection.

b. **Morphological identification of ticks:** Out of 15 ticks collected 8 ticks were ornate and 7 ticks were inornate ticks. The present study confirmed the ornate ticks as *Amblyomma gervaisi*and inornate ticks as *Amblyomma pattoni*. The morphological features of *Amblyomma gervaisi* were ornate scutum having five creamy yellow spots with one at the centre and the others at edge of the scutum in male ticks, and a heart-shaped scutum with three creamy yellow spots, one at the posterior end and other two at lateral side of the scutumin female ticks (Fig. 1). The ticks were having 11 numbers of festoons in the posterior margins (Fig. 2). The mouthpart was longirostrate with blade like chelicerae (Fig. 3) and 3:3 hypostomal dentition (Fig. 4). Comma-shaped spiracular openingswas presentat the 4th coxal region (Fig. 5).
Amblyomma pattoni was identified based on the morphological characters like inornate scutum (Fig. 6) with longirostrate mouth parts accompanied with blade like chelicerae (Fig. 8) and 3:3 hypostomal dentition (Fig. 9) as like *Amblyomma gervaisi*. In addition, spur-like prominence were found at the posterior margins of the coxae (Fig. 10) and the spiracular opening at the 4th coxae (Fig. 11).The genital opening was positioned in median line at the level of second coxa beneath the basis capitulum (Fig. 12).

c. **Morphological and Molecular characterization of *Haemoproteus* sp.:** Giemsa's stained blood smears revealed the presence of intracytoplasmic halter shaped gamonts of *Haemoproteus* sp. which partially encircled the nucleus of the RBC (Fig. 13). The isolated DNA amplified with genus specific primers revealed 523 bp amplicons which confirmed the rRNA gene of *Haemoproteus* sp., (Fig. 14).

Discussion

Among theixodid group of ectoparasites, morphological description of *Amblyomma pattoni* was reported by Neuman (1910) based on that the present study confirmed the presence of *A. pattoni* in the rat snake. *Amblyomma* (*Aponomma*) *gervaisi* species have previously been reported in rat snakes, cobras, and pythons in southern India (Soundararajan *et al.* 2013; Catherine *et al.* 2017) [14, 1]. Further detailed electron microscopic morphological description of *A. gervaisi* was studied by Soundararajan (2020) [2] which provided a morphological key for the identification of the *A. gervaisi*.In our findings, the basis capitulum of the *A. gervaisi* was dorso-ventrally flask-shaped which had entire mouth parts, comprising a mandible, hypostome, and two pedipalps which were in agreement with the observations given by Ghosh & Misra (2012) [4]. As the location of ticks on the body of the snake was in ventral abdomen (Rosenthal, 1997) [12], beneath the scales of the snake (Catherine *et al.*, 2017) [1], similarly our study also found *Amblyomma* ticks on the dorsal surface and beneath the scaly skin of the rat snake. Due to the presence of tick infestation, the scales were elevatedin our case, which emphasizes that the elevated scales may suspected for the hidden ticks.

Previous studies by Mader (1996) [8] and Catherine *et al.*, (2017) [1] have emphasised the skin disorders in snakes, such as dermatitis, dysecdysis and lumps, but neither a single observation was detected in the present investigation. The *Amblyomma* genus has been highlighted as one of the potential threats to domestic and wild animal populations with a special reference to public health (Guglielmone *et al.* 1992) [5]. Further research is necessary to determine the extent to which the ticks may transmit diseases to animals as well as humans in India.

Telford (2009) [15] discovered distinct blood protozoan *Haemoproteus* species, *Haemoproteus mesnili* and *Haemoproteus balli* in Egyptian and spitting cobras, respectively. According to Laison and Naiff (1998) [7] and Ursula *et al.* (2014) [16], leeches could act as a vector for hemoparasites in reptiles, whereas Zhang *et al.* (2014) [19] reported that biting midges louse flies and ticks may transmit *Haemoproteus* sp., during blood feeding. Initial haematological study by Vairamuthu *et al.*, (2010) [17] reported the *Hepatozoon* sp. like infection in nucleated erythrocytes of snake from Arignar Anna Zoological Park, Chennai, Tamil Nadu, India. In general, *Haemoproteus* species is believed to be less harmful and stress may be to hold accountable for the growth and propagation of the parasitic infection in reptiles. It has been proposed that *Haemoproteus* infections may persists chronic for an entire life, which may be life threatening (Mader, loc cit).

However, unlike *Plasmodium*, *Haemoproteus* infection is not known to result in haemolytic anaemia (Jacobson, loc cit) except in severe cases leading to death of reptiles (Miller and Fowler, 2015) [9]. Veeraselvam *et al.*, (2018) [18] reported intra-erythrocytic gamonts of *Haemoproteus* sp., in a rescued rat snake with the haematological parameters from Thanjavur region of Tamil Nadu. This study also coincides with the findings of Veeraselvam *et al.*, (2018) [18] and the blood protozoan *Haemoproteus* sp., was confirmed by molecular analysis.

The rat snake was treated with Ivermectin (0.2 mg/kg) to combat the ecto-parasitic infection and also treated with Chloroquine (15 mg/kg) orally for 10 days. This study is the first molecular investigation of *Haemoproteus sp*. in a rat snake from India with the report of *Amblyomma pattoni* in rat snakes from Southern Region of India.

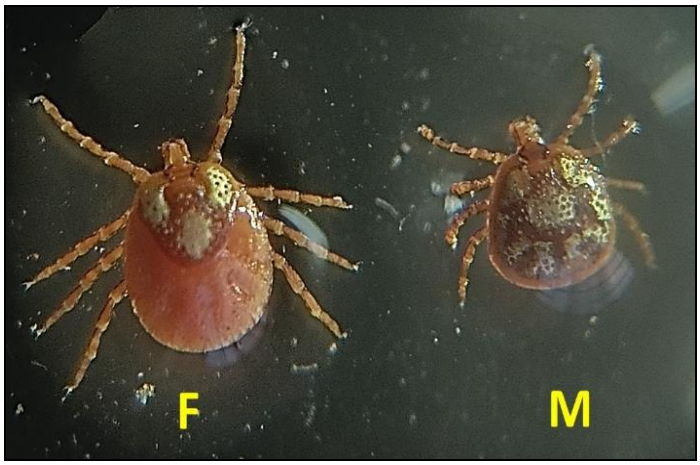

Fig 1: Gross view of *Amblyommagervaisi* – Female and Male (Ornate scutum)

Fig 2: Xylene cleared *Amblyomma gervaisi* (40x)

Fig 3: Mouth part of *Amblyomma gervaisi* with blade like chelicerae (100x)

Fig 4: Mouth part of *Amblyomma gervaisi* - 3:3 hypostomal dentition (400x)

Fig 5: *Amblyomma gervaisi* – Spiracular opening at the 4th coxal region (100x)

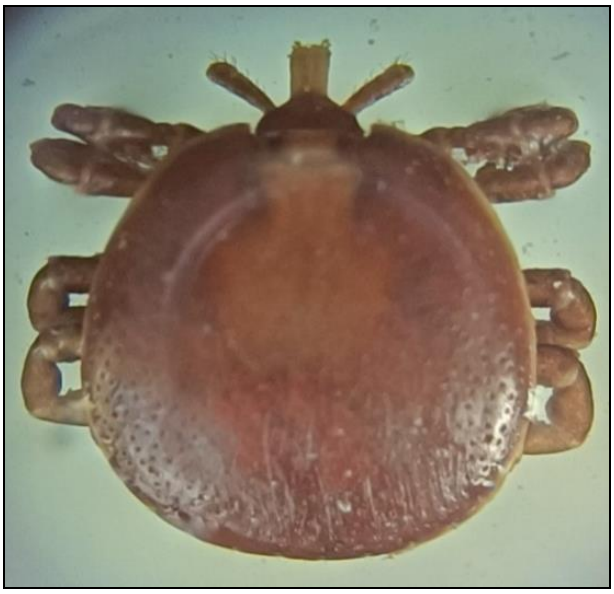

Fig 6: Gross view of *Amblyomma pattoni*– Inornate scutum

Fig 7: Xylene cleared *Amblyomma pattoni* (40x)

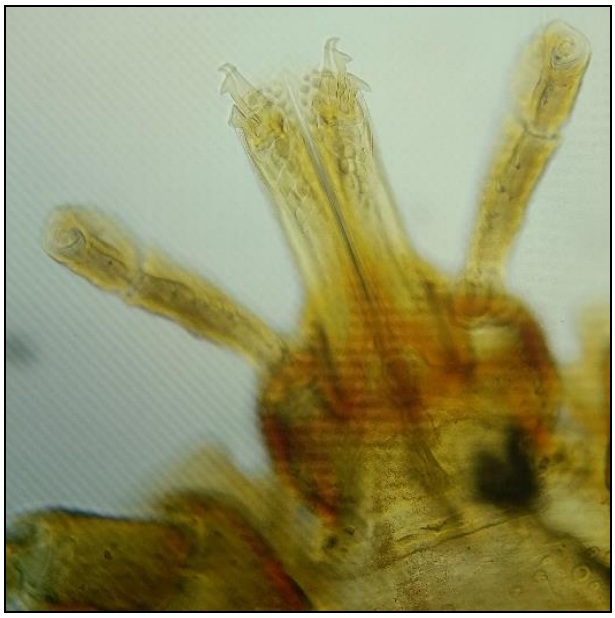

Fig 8: Mouth part of *Amblyomma pattoni* with blade like chelicerae (100x)

Fig 9: Mouth part of *Amblyomma pattoni* - 3:3 hypostomal dentition (400x)

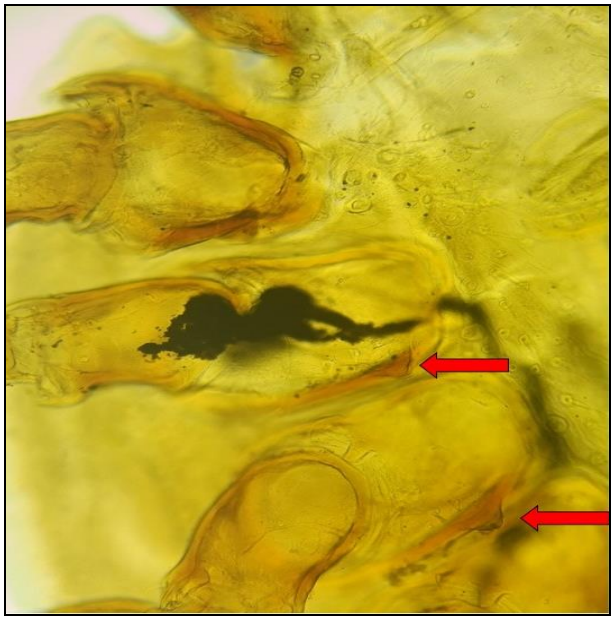

Fig 10: *Amblyomma pattoni* – Coxal spur (400x)

Fig 11: *Amblyomma pattoni* – Spiracular opening at the 4th coxal region (100x)

Fig 12: *Amblyomma pattoni* – Genital orifice (100x)

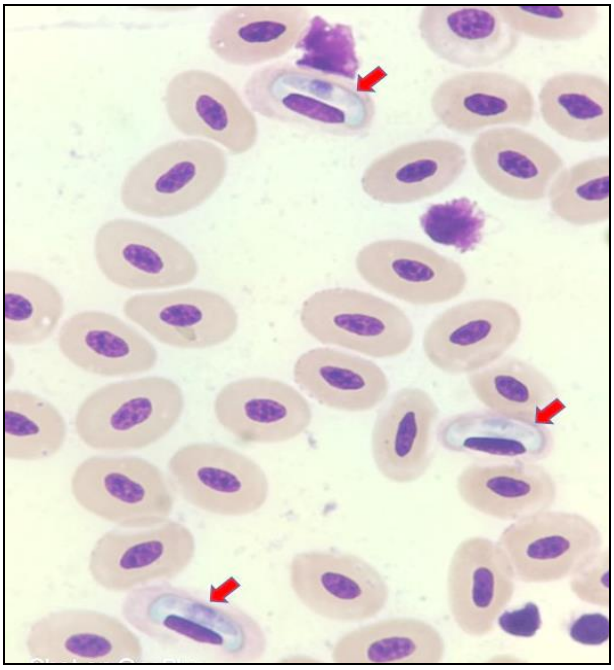

Fig 13: Halter shaped gamonts of *Haemoproteus* sp., in the nucleated erythrocytes

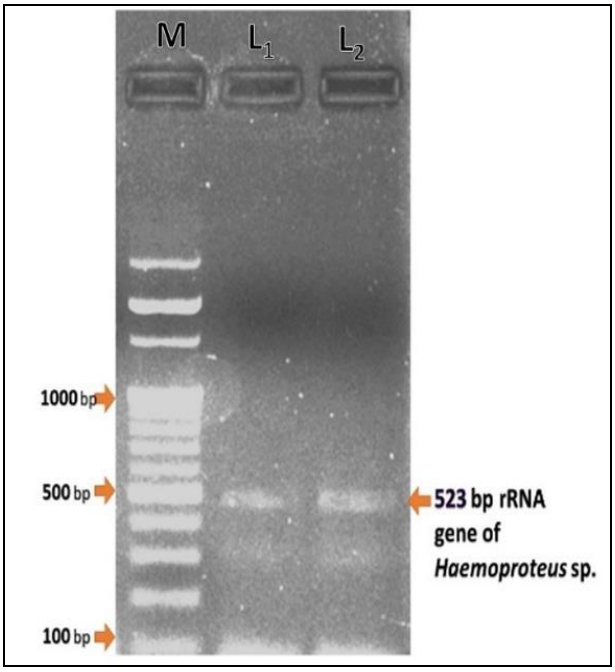

Fig 14: PCR amplification of 523 bp rRNA gene of *Haemoproteus* sp.

Table 1: The haematological and biochemical analysis of the blood sample revealed the following parameters

Parameters	Values
Haemoglobin (Hb)	9.8 g/dL
RBC Count	5.9 X 10^6/µl
PCV	33%
WBC Count	29.5 X 10^3 /µl.
BUN	34 mg/dl
Creatinine	0.88 mg/dl
Bilirubin (Direct)	0.88 mg/dl
Glucose	69 mg/dl
Cholesterol, and	168 mg/dl
ALT	52 mg/dl
SGOT	199.6 mg/dl

Conflict of interest

The authors do not have any conflict of interest for this article.

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Conclusion

The examination of the snake revealed signs of anaemia and infection, with haematological and biochemical analyses supporting these findings. The presence of Amblyomma gervaisi and Amblyomma pattoni ticks was morphologically confirmed, with ticks found both dorsally and ventrally on the snake's body. While skin disorders commonly associated with tick infestation were not observed, the potential threat of Amblyomma ticks to animal and human health warrants further investigation. The identification of Haemoproteus sp. in the snake's blood, confirmed both morphologically and through molecular analysis, suggests a parasitic infection, although unlike Plasmodium, it is not typically associated with haemolytic anaemia. Treatment with Ivermectin and Chloroquine was administered to combat the ecto-parasitic infection. This study marks the first molecular investigation of Haemoproteus sp. in a rat snake from India and reports the presence of Amblyomma pattoni in rat snakes from the Southern Region of India. Further research is needed to understand the transmission dynamics and potential health implications of these findings for both animals and humans in India.

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