Prevalence and molecular characterization of Haemoproteus tinnunculi from falcons in Saudi Arabia

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ABSTRACT

Objective: This study aimed to inspect the occurrence of Haemoproteus tinnunculi (H. tinnunculi) in falcons from the central area of Saudi Arabia.

Materials and Methods: Blood samples from 100 falcons species, including 55 Falco cherrug, 22 Falco peregrinus, 13 Falco pelegrinoides, and 10 Falco rusticolus, were collected from November 2018 to April 2019 and examined for H. tinnunculi by microscopic examination and nested PCR, targeting a cytochrome b (cytb) gene.

Results: The prevalence was 1% by microscopic examination. The prevalence rate of H. tinnunculi was 1% by the microscopic method and 3% by PCR. Only F. cherrug was infected. In the sequence and phylogenetic analyses, the two cytb H. tinnunculi sequences were 100% identical and closely related to the Lithuanian isolate with 99.33% identity.

Conclusions: This study presents the first report of molecular detection and characterization of H. tinnunculi in F. cherrug from the Kingdom of Saudi Arabia.

Introduction

Haemoporida parasites infect a diversity of avian groups and are transmitted by blood-sucking insects. There are three main genera (Haemoproteus, Plasmodium, and Leucocytozoon) identified in birds, and each genus has many species [1–3]. Haemoproteus species are worldwide prevalent. Although climate, vector activity, and bird migration are risk factors associated with distributing these blood parasites within or between temperate, subtropical, and tropical regions, they are diverse in tropical countries [1,4–7].

In Germany and the USA, avian hemoparasites have been reported in many raptors [8,9]. In Kingdom of Saudi Arabia (KSA), although Plasmodium and Haemoproteus parasites were recorded in the Skink lizard and saker falcons, respectively [10,11], their prevalence is rare. The genus Haemoproteus includes 128 species, mostly pathogenic in domestic birds, leading to various clinical signs, such as vomiting, depression, and tremors [12–14].

Recently, Haemoproteus tinnunculi (H. tinnunculi) has been diagnosed in falcons in many places in the world [14,15]. The pathogenicity of this hemoparasite was recognized in falcons from Kuwait. The clinical signs were poor appetite, weight loss, wing arthritis, vomiting, ataxia, swollen and closed eyes, and lethargy [15].

Recently, polymerase chain reaction (PCR) has been used successfully to diagnose blood parasite infections and provides more sensitivity and accuracy than microscopic examination. Moreover, DNA sequencing helps to identify the closely related parasites and their evolutionary [7,16–18]. The sequence analysis of mitochondrial cytochrome b (cytb) was used for the genetic characterization of bird haemosporidian species [7,17,19]. Therefore, this study aimed to identify H. tinnunculi by PCR in captive falcons and to study the

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genetic characterization of the circulating *H. tinnunculi* by DNA sequencing and phylogenetic analysis of the *cytb* gene.

**Materials and Methods**

**Sampling**

This study was performed at Riyadh and Qassim Provinces in the central region of KSA (Fig. 1) from November 2018 to April 2019. One hundred captive falcons consisting of 55 *Falco cherrug*, 22 *Falco peregrinus*, 13 *Falco pelegrinoides*, and 10 *Falco rusticolus* were collected and examined for the detection of *H. tinnunculi* infection. Blood samples (0.5 ml) were taken from the brachial or jugular vein of each falcon after being anesthetized with isoflurane into ethylenediaminetetraacetic acid (EDTA) tubes for further analysis.

**Blood smear**

Three thin smears were made from each falcon with a drop of fresh blood on the glass slide, air dried, then fixed in absolute methanol for 15 min, then stained by Giemsa stain (freshly diluted 1:10 with dH₂O) for 10–15 min, and then examined using the 10× lens power, then under the oil immersion lens (100×) to find *H. tinnunculi*.

**DNA extraction and PCR amplification**

The total parasitic DNA was extracted from blood samples using DNeasy Blood and Tissue Kit (QIAGEN, Beckman Instruments, Inc.), according to the protocol of the manufacturer. Briefly, add 10 µl of anticoagulated blood and proteinase K (20 µl) into a microcentrifuge tube (1.5 ml), and

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**Figure 1.** The location of the central region of Saudi Arabia that was sampled.
add Phosphate buffered saline (PBS) to a final volume of 220 µl. Add buffer AL (200 µl), without ethanol, into each blood sample, vortexing, and then incubate at 56°C for 10 min. Add ethanol 96%–100% (200 µl) to the specimen, and mix well using a vortex. DNA samples (200 µl) were taken after loading the kit’s spin column. The aliquots of DNA were kept at −20°C till used with PCR. The mixture of PCR (25 µl) included GoTaq® Green Master Mix 2X (15 µl), 1 µl isolate (MK580171), isolated from the central zone of Saudi Arabia, from one host (1.5%) and as high as two falcons (2.9%) by using PCR. Similarly, the prevalence of the infection in F. cherrug was high by PCR (5.5%) compared to the microscopy (1.8) (Table 1).

Sequence homology and phylogenetic analysis
We identified two identical sequences (100% identical in their partial sequences of the cytb gene), almost contained lineage belonging to H. tinnunculi, from one host species (F. cherrug) at the central zone of Saudi Arabia, one from Riyadh (MN780909), and the other from Qassim (MN780908). Sequences were closely related to H. tinnunculi isolate (MK580171), isolated from F. subbuteo, Lithuania with a node support value of 61, but genetically differ only in 0.01 in their partial sequences of the cytb gene (Figs. 2 and 3). The neighbor-joining method using the data of nucleotide sequences targeted the cytb gene showed two tight clusters (Cluster 1 and 2; Fig. 3). Cluster 1 is separated into two clades (clades A and B). Clade A represents Haemoproteus species from varied birds found in this study and other Asian, European countries, such as Lithuania, Spain, Norway, Germany, and Iran, besides sequences from Mexico and the USA. It was noted that the sequences of most of the clade A sequences were not identified at the level of parasite species, except Harmochirus brachiatius (MK580170) from Lithuania and H. tinnunculi

**Table 1.** Prevalence of H. tinnunculi in falcons from Riyadh and Qassim.

| Variables               | Blood film +ve n, (%) | PCR +ve n, (%) |
|-------------------------|-----------------------|----------------|
| Region                  |                       |                |
| Riyadh (n = 32)         | 0                     | 1 (3.1)        |
| Qassim (n = 68)         | 1 (1.5)               | 2 (2.9)        |
| Falcon species          |                       |                |
| F. cherrug (n = 55)     | 1 (1.8)               | 3 (5.5)        |
| F. peregrinus (n = 22)  | 0                     | 0              |
| F. pelegrinoides (n = 13)| 0                    | 0              |
| F. rusticolus (n = 10)  | 0                     | 0              |
| Total (n = 100)         | 1 (1)                 | 3 (3)          |
from Saudi Arabia and Lithuania. Clade B represents *Plasmodium* sp. sequence from Benin.

It is worth noting that the parasites of clade A have a low genetic divergence (0.01–0.03) in their partial *cytb* gene sequences, indicating their close relationship, and the genetic variation was up to 0.08 when considered the comparison with clade B sequence. The study showed the first molecular detection and characterization of *H. tinnunculi*.
parasitize *F. cherrug* and grouped with *Haemoproteus* and *Parahaemoproteus* species in cluster 1, suggesting their close relationship appeared as a sister targeting the *cytb* gene.

**Discussion**

In KSA, *H. tinnunculi* was reported in 2001 and 2010, and the parasite was detected by microscopy among Saker falcons, as reported in Riyadh at Fahad Bin Sultan Falcon Center [11,22]. However, the current study is the first in the central region of KSA using a molecular approach to diagnose *H. tinnunculi* parasitizing falcons. This study revealed a very low prevalence of *H. tinnunculi* by microscopic inspection of blood smears (1%) and PCR (3%) among 100 examined falcons. The low prevalence of *H. tinnunculi* in this study is relatively similar to the previous reports in Middle East countries: 3.8% in Kuwait [15] and 5.3% and 6.7% in UAE [11,23], and it was significantly lower than reported in KSA (81%) in 2010 [22]. The difference in prevalence rates can be attributed to differences in the level of parasitemia, sampling timing, handling, geography, health status, behavior, and management provided for falcons.

The molecular studies about *H. tinnunculi* infection in falcons are rare worldwide due to difficulty in obtaining and maintaining sporozoites [7], whereas a smear-based diagnosis is essential; however, it remains insufficient or sometimes unreliable in determining and diagnosing of *Haemoproteus* species [24]. In the present study, molecular detection was higher than blood smears. Previous studies have indicated comparable high sensitivity to PCR when compared to microscopy for the diagnosis of avian *Haemoproteus* or *Malaria* [18,25,26]. Further, the higher sensitivity of PCR indicates their availability to detect the infection in contrast to microscopy and to reduce possible bias in estimating the prevalence of avian blood parasites [7,27,28].

Interestingly, in this study, the infection was detected and confirmed only in *F. cherrug*, whereas infection was not detected in other falcon species by blood smears and PCR. This finding indicates that the correlation between *F. cherrug* and *H. tinnunculi* infection was positive, with an increased risk of *H. tinnunculi* infection in this falcon species. Rahim et al. [11] have studied only one falcon species (*F. cherrug*) in Riyadh at Fahad Bin Sultan Falcon Center. This study did not focus on other different species of falcons. Furthermore, Naldo et al. [22] have been examined the infection among different species of falcons. Still, the study did not focus on whether all species were infected with *H. tinnunculi*.

The inability to detect *H. tinnunculi* in *F. peregrinus*, *F. pelegrinoides*, and *F. Rusticolus* is contrast with the previous studies that reported *H. tinnunculi* infection in *F. Peregrinus* and *F. rusticolus* from Kuwait and *Falco sparverius* from Pennsylvania [15,29]. This inconsistency in results is unexpected, suggesting that *F. cherrug* in this study regions may have more exposure than other falcons or may be due to differences in the host species concerning *H. tinnunculi* infection. Meixell et al. [27] concluded that host-specific vectors might be affected by several factors such as vector exposure, host body size (larger size attracts more insects), and plumage color (bright color attracts more insects). Hence, further studies are needed with more samples from these falcons to clarify their role in the epidemiology of *H. tinnunculi* in the KSA.

A partial sequence analysis of the *cytb* gene supplies the insights of phylogenesis and differentiates between different families, genera, and subgenera as well as taxonomic biodiversity and genetic divergence of haemosporidians [7,17].

Alignment of nt sequence of the *cytb* gene showed that *H. tinnunculi* isolates from Saudi are 100% identical and closely related (99.35%) to *H. tinnunculi* isolates from Lithuania with a genetic divergence of 0.01%. This finding indicates that *H. tinnunculi* isolates undergo low genetic divergence over the partial sequences of the *cytb* gene. A previous study compared genetic differences between the apicoplast gene and the *cytb* gene and found that the genetic differences in the sequence of the *cytb* gene are less than that of the apicoplast gene sequence in closely related haemosporidia [7]. In another study, genetic divergence in the *cytb* gene sequence between *Haemoproteus iwa* and *Haemoproteus jenniae* was little (0.6%) and up to 4% when *clpc* gene was considering [30].

The *cytb* gene phylogeny (Fig. 3) confirmed the close relation of Saudi *H. tinnunculi* to Lithuanian *H. tinnunculi* isolate with 62% of nodal support. These sequences constitute first reference sequences for *H. tinnunculi* species from Saudi Arabia. Furthermore, the tree showed that Saudi isolates are clustered with other *Haemoproteus* species from Lithuania, Spain, Norway, Germany, Iran, in addition to sequences from Mexico and the USA with sequence similarities ranged from 92% to >99%. On the other hand, the phylogenetic tree showed that the parasites of clade A are closely related with nodal support of 98% with genetic divergences of 0.03%, indicating possibly the same evolutionary ancestor and transmission by the same vector (biting midges).

**Conclusion**

This is the first study that uses the molecular characterization of *H. tinnunculi* that infects *F. cherrug*. The phylogenetic analysis of the *cytb* gene sequence of *H. tinnunculi* isolate of Saudi origin showed a close relation to Lithuanian.
isolate. There are no cytb gene sequence data for *H. tinunculi* from KSA other than the sequences mentioned here. Thus, these findings call for more studies on a larger scale to provide further molecular characterization and to know the relationship between *H. tinunculi* and the different species of falcons.

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**Conflicts of interest**

The authors declare that they have no conflicts of interest.

**Authors’ contribution**

Alfaleh F. and Alyousif M.: conceptualization, methodology, investigation, data curation, writing—original draft, and writing—review and editing, Elhaig M.: sequencing and phylogenetic analyses, writing—original draft, and writing—review and editing.

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