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ORIGINAL ARTICLE

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 99.35% identity.
Conclusions: This study presents the first report of molecular detection and characterization of *H. tinnunculai* in *F. cherrug* from the Kingdom of Saudi Arabia.

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Introduction

Haemosporida 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 dH_2O) 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

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 (20 pmol) of each of the primers, 100 ng of extracted DNA, and free nuclease water to 25 µl final volume. The identification and characterization of *Haemoproteus* species were performed using a nested polymerase chain reaction (PCR) to amplify a *cytb* gene by the following primers: HaemNFI: 5′-CAT ATA TTA AGA GAA ITA TGG AG-3′) and HaemNR3: 5′-ATA GAA AGA TAA GAA ATA CCA TTC-3′) for first PCR and HAEMF: 5′-ATG GTG CTT TCG ATA TAT GCA TG-3′ and HAEMR2: 5′-GCA TTA TCT GGA TGT GAT AAT GGT-3′ for second PCR, as previously described [20,21]. The PCR profile included a 5-min initial denaturation at 94°C, followed by 35 cycles of incubation at 49°C for 30 sec, 55°C for 30 sec, and 72°C for 45 sec with a final extension at 72°C for 10 min. The products of PCR were analyzed using electrophoresis via agarose gel (1.5%) containing 0.5 µg/ml ethidium bromide, and the image was taken by using a gel documentation system (Upland, CA).

DNA sequencing and phylogenetic analysis

The PCR targeting *cytb* gene (478-bp) was purified and sequencing in an automated DNA sequencer (ABI 3730XL, Solgent Co. Ltd., South Korea). The sequence nucleotides (nt) were read by DNA BaserV3 software, and a blastN search was performed using the Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/BLAST) to identify the *cytb* region of the mtDNA entries in the GenBank database, with the highest nt sequence identities. An evolutionary analysis was inferred by using the neighbor-joining method. The analysis included 29 nt sequences of the *cytb* gene. There were a total of 505 positions in the final dataset. An evolutionary analysis was conducted in MEGA X. The partial sequences (n = 2) identified in the study were deposited in the GenBank under accession number MN780908 and MN780909. Besides, the divergence between the partial sequences of *cytb* gene of *Haemoproteus* was evaluated using the maximum composite likelihood model and a bootstrap procedure (1,000 replicates) as performed in MEGA X.

Results

Detection of H. tinnunculi by stained blood smears and PCR

The infection with *H. tinnunculi* was detected in one blood sample collected from *F. cherrug* by microscopic examination of blood smears, giving a prevalence rate of 1% (1/100) and up to 3% (3/100) by a PCR targeting of the *cytb* gene. One of these was positive by PCR and microscopy. The infection was confirmed in *F. cheerug*, whereas the other falcons were tested negative by both tests. One falcon from Riyadh showed the infection by PCR with phylogenetically unique *H. tinnunculi* (prevalence 3.1%), whereas all falcons were negative by blood smear examination (Table 1). On the other hand, one falcon from the Qassim region was found infected by using microscopy with morphologically and phylogenetically unique *H. tinnunculi* (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 nodel 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 brachiatus* (MK580170) from Lithuania and *H. tinnunculi*

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

Variables	Prevalence	
	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)

	MK580171	MN780908	MN780909	GQ141558	MK135927	KF309188
MK580171 H.tinnunculi		0.00	0.00	0.02	0.04	1.45
MN780908 H.tinnunculi	0.01		0.00	0.02	0.04	1.5
MN780909 H.tinnunculi	0.01	0.00		0.02	0.04	1.5
GQ141558 Parahaemoproteus species	0.03	0.03	0.03		0.05	1.41
MK135927 Plasmodium species	0.08	0.08	0.08	0.08		1.52
KF309188 Leucocytozoon quynzae	0.26	0.26	0.26	0.25	0.26	

Figure 2. Divergence table of *cytb* gene of *H. tinnunculi* isolates in this study with previously published *Haemoproteus* nucleotide sequences.

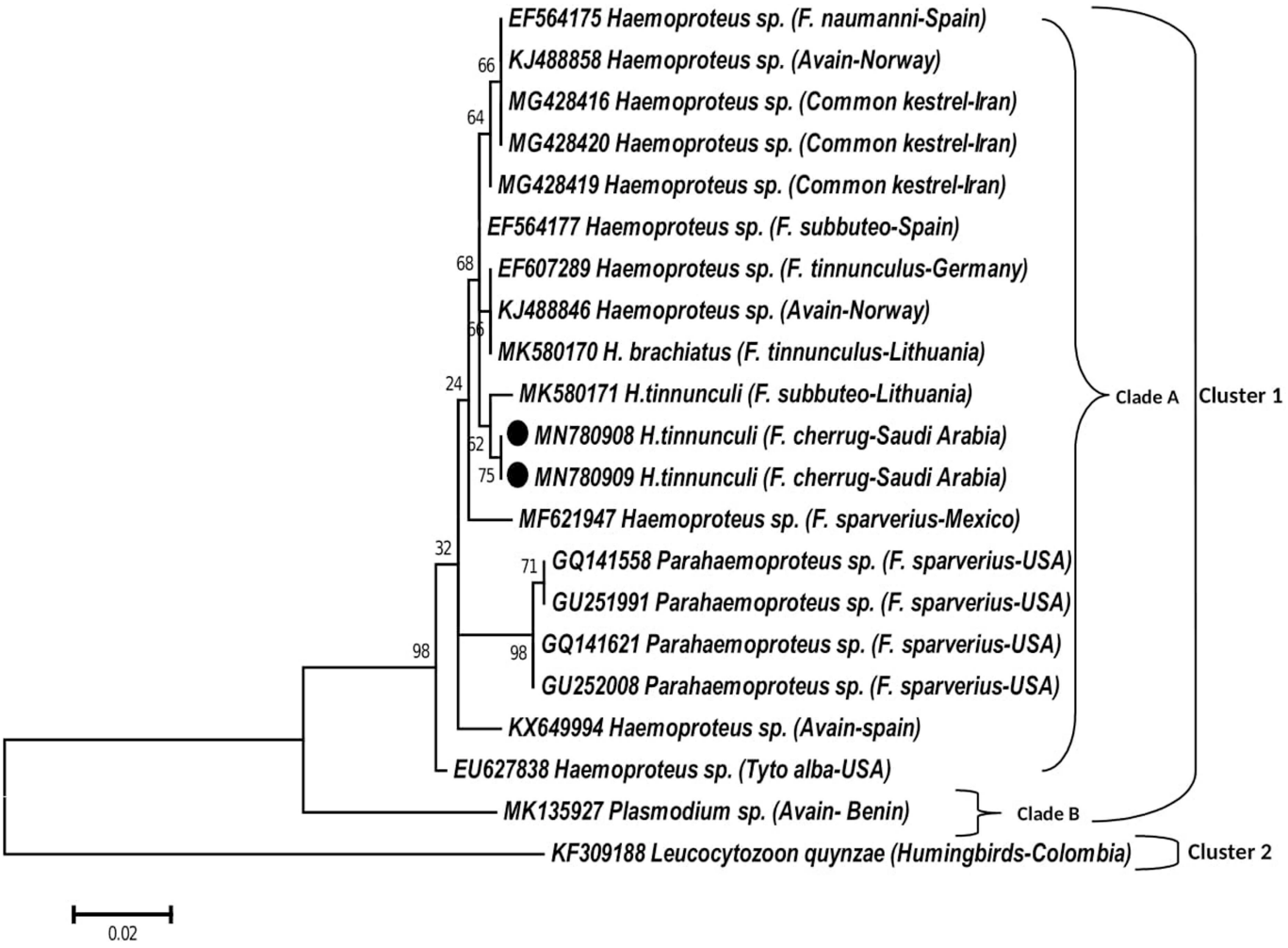

Figure 3. Phylogenetic analysis of *cytb* gene of *H. tinnunculi* with other *Haemoproteus* sequences from GenBank based on *cytb* gene. The bootstrap test maximum likelihood method in MEGA X (1,000 replicates) was used to draw the tree. *H. tinnunculi.*

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. tinnunculi* 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. tinnunculi* 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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