Exo-erythrocytic stages of *Haemoproteus* sp. in common buzzard (*Buteo buteo*): A histopathological and molecular study

Hamidreza Shokrani a, *, Hassan Norouzian b, Omid Dezfoulian a

a Department of Pathobiology, Faculty of Veterinary Medicine, Lorestan University, Khorramabad, Iran
b Department of Clinical Sciences, Faculty of Veterinary Medicine, Lorestan University, Khorramabad, Iran

ARTICLE INFO

Keywords:
Haemosporidians
Leucocytozoon
Co-infection
Cytochrome b
Phylogenetic analysis

ABSTRACT

Haemosporidian parasites are responsible for anemia, acute tissue degeneration, and depopulation in wild birds. This study aimed to investigate the prevalence of haemosporidians and also morphologic and molecular evaluation of tissue stages of *Haemoproteus* sp. in common buzzards (*Buteo buteo*). Eleven free-living common buzzards were referred to the Avian Clinic of Veterinary School of Lorestan University with lethargy, weight loss, and ataxia. Gametocytes of *Leucocytozoon buteonis* were found in blood smears of six (54.5 %) birds, while one had simultaneous infection with blood stages of *Haemoproteus* and *Leucocytozoon*. During histopathological examinations, exo-erythrocytic stages of the genus *Haemoproteus* were seen in the lung and kidney of a dead bird. This study is the first report of exo-erythrocytic infection of *Haemoproteus* in common buzzards. Molecular assays confirmed the infection of *Haemoproteus* sp. (lineage BUTBUT15) in tissue samples. Phylogenetic analysis using cytochrome b gene suggested that BUTBUT15 was more closely related to the lineages isolated from the family Falconidae in contrast to the Accipitridae.

1. Introduction

Haemosporidians (Phylum: Apicomplexa; Order: Haemosporida) are obligate intracellular protozoan parasites which infect a variety of reptilian, avian, and mammalian hosts (Borner et al., 2016). Little information is available about tissue development, morphological diversity, and the possibility of tissue destruction of avian haemosporidians. On the other hand, the life cycle of hemoproteids (Haemosporida: Haemoproteidae), particularly exo-erythrocytic stages, has been less described than other haemosporidians (Valkiunas and Iezhova, 2017).

Diagnoses of haemosporidian infections are made primarily based on the presence of micro- or macrogametocytes in blood smears. However, in low parasitemia, it is extremely difficult to detect the blood stages of *Haemoproteus* spp. by microscopy (Valkiunas, 2005). Previous studies indicate that the erythrocytic stages of haemosporidians have been more recognized as compared to exo-erythrocytic stages, especially in raptors (Munoz et al., 1999; Krone et al., 2001; Svorčík and Švobodová et al., 2015). Due to similarities between the tissue stages of haemosporidians, application of molecular methods in parallel with histopathology is inevitable, especially in mixed infections with two or more parasites (Cardona et al., 2002; Ferrell et al., 2007; Donovan et al., 2008; Olias et al., 2011; Pacheco et al., 2011; Cannell et al., 2013; Palinauskas et al., 2013; Valkiunas and Iezhova, 2017).

The risk and genetic diversity of haemosporidian infections in different wild migratory birds are probably greater than those of other bird species. These birds are principally susceptible to be infected with blood parasites. They can play significant roles in the ecology and circulation of some haemosporidians (Hubalek, 2004; Valkiunas, 2005; Nourani et al., 2020). Common buzzard (*Buteo buteo*) is a protected bird species that belongs to the family Accipitridae (Order: Accipitriformes) migrates through Iran during spring and fall (Mansoori, 2013). No study has been conducted to describe the prevalence of haemosporidians in Iranian birds of prey. The present study aimed to investigate the prevalence of haemosporidians in free-living common buzzards from western Iran, and also to evaluate the tissue stages of *Haemoproteus* sp. on the basis of histopathological and phylogenetic findings.

* Corresponding author. Faculty of Veterinary Medicine, Lorestan University, Khorramabad, Iran.
E-mail addresses: shokrani.hm@lu.ac.ir, hamidreza_shokrani@yahoo.com (H. Shokrani).
2. Materials and methods

2.1. Study area and blood sampling

Eleven free-living adult common buzzards were referred to the Avian Clinic of Veterinary School of Lorestan University between February 2017 and April 2020. All birds were collected in Lorestan province (33°48’N, 48°35’E), West of Iran. Lethargy, weight loss associated with anorexia and poor appetite, ruffled feathers, and incoordination were observed in clinical examinations of all birds. According to the statements, these birds had no history of alimentary, respiratory, or other pathognomonic signs.

Two smears were prepared with blood collected from the brachial vein of each bird. Smears were air-dried, fixed in 100 % methanol, and stained with Giemsa as described by Valkiunas (2005). Each sample was microscopically checked for at least 30 min at × 400 magnification. Positive samples were also examined at × 1000 magnification. The intensity of infection was estimated as a percentage by counting the number of gametocyte host cells per 10,000 erythrocytes. The species of the genus Leucocytozoon was identified using the taxonomic keys following Valkiunas (2005) and Valkiunas et al. (2010).

2.2. Necropsy and tissue sampling

In March 2017, a male common buzzard with a gunshot wound on his wing died shortly without any chance of treatment. The bird had co-infection with Leucocytozoon and Haemoproteus gametocytes. A routine necropsy was performed and internal organs including the brain, liver, lung, kidney, spleen, heart, and intestinal tract were removed. Subsequently, 1 g of each tissue sample was transferred to a 1.5 ml sterile tube and stored at −20 °C until DNA extraction. The rest of tissues were fixed in neutral buffered formalin 10 % and embedded in paraffin wax. The 4–5 μm thick sections were stained with hematoxylin and eosin (Valkiunas, 2005). The sizes of meronts and merozoites found in this study were expressed in micrometers (μm) as mean ± standard deviation (SD) followed by the range in parentheses. The number of meronts was counted in all available high-power fields (HPFs; × 400 magnification) and the result was shown as average number per 10 HPFs.

2.3. DNA extraction, PCR, and sequencing

Tissue samples were thoroughly powdered under liquid nitrogen. DNA extraction was performed on 25 mg of each tissue sample using the YTA Genomic DNA Extraction Mini Kit (Yeka Tajhiz Azma, Iran) following the supplier’s instructions. PCR reaction was performed as previously described using specific primers HAEMF (5′ATGG TGCTTTCCGATATATGCATG3′) and HAEMR2 (5′GCGTTACCT GCATGGATAATGCATG3′) to amplify a 478bp fragment of the conserved regions of the cytb gene (Bensch et al., 2000).

Samples showing positive amplifications were selected for sequencing. The PCR products were purified and sequenced with both forward and reverse primers by Sequtech (Mountain View, CA). The obtained sequence was blasted in MalAvi database (Bensch et al., 2009). Pairwise sequence alignments were performed by the ClustalW (MEGA X program, version 10.2.5) between the obtained sequence and the other sequences available on MalAvi. A phylogenetic tree was constructed using MEGA X program with the maximum-likelihood method (Tamura et al., 2011). The degree of similarity between the studied Haemoproteus sp. and GenBank (accession no. MG428417) was 97–98 % identical to lineages recorded in Lesser kestrel (Falco naumanni) (lineage LK02) and other Falco spp. (lineages LK03 and LK04). In BLAST search, the nucleotide sequence of the cytb gene of the studied Haemoproteus sp. (BUTBUT15) was 97–98 % identical to lineages recorded in Lesser kestrel (Falco naumanni) (lineage LK02) and other Falco spp. (lineages LK03 and LK04). The degree of similarity between the studied Haemoproteus sp. and BUTBUT15 was 98 % and 97 % identical to H. brachiatius (lineage LK03) and H. tinnuculi (lineage FALSUB01) obtained from Falco.
Diurnal raptors from Accipitridae are highly parasitized by leucocytozoids (Walther et al., 2016). Epidemiological studies have indicated that Leucocytozoon infection in birds from Accipitridae is ten times more common than Haemoproteus infection in birds from Falconidae (Valkiunas, 2005). Our microscopic results indicate a high prevalence of L. buteonis (54.5 %) in free-living common buzzards. The high prevalence of L. buteonis in apparently healthy adult birds suggests that this is a harmless endemic haemoparasite of common buzzards in west of Iran.

Some avian Haemoproteus spp. create acute illness and intensive mortality through exo-erythrocytic meronts (Valkiunas and Iezhova, 2017). Evidence suggests that the exo-erythrocytic stages might be different in their manifestations in the avian hosts, which could probably be dependent on various factors, including host diversity, new emergences of the parasite, and infection of birds with unspecified strains. Olias et al. (2011) reported a parakeet infected with H. minutus that produced deadly megalomeronts while parasitemia was absent. In this regard, it has been propounded that many haemosporidian strains have different developmental stages in the aberrant hosts. Tissue stages can develop in these hosts, but these structures will be arrested where the erythrocytic stages do not appear (Valkiunas and Iezhova, 2017).

Prior to the availability of molecular and genetic phylogenies, there was little information about the exo-erythrocytic stages of haemoproteids, and the identification was mainly based on morphological characteristics. If genetic lineages of haemosporidian parasites are linked with morphospecies, a remarkable volume of information would be available for phylogenetic analysis of these blood parasites (Valkiunas and Iezhova, 2017). In the present study, exo-erythrocytic stages of the genus Haemoproteus were identified in the histological sections. The prevalence rate of leucocytozoids in raptors from different regions is more related to the avian species, climatic conditions, distribution of insect vectors, and also the methods applied for diagnosis (Walther et al., 2016; Nourani et al., 2020). There is a tendency for a higher prevalence of the L. toddi group in the direction from the tropical latitudes to the middle latitudes of the Northern Hemisphere (Valkiunas, 2005). Our microscopic results indicate a high prevalence of L. buteonis (54.5 %) in free-living common buzzards. The high prevalence of L. buteonis in apparently healthy adult birds suggests that this is a harmless endemic haemoparasite of common buzzards in west of Iran.

Table 1

| Feature                        | Measurements (µm) |
|--------------------------------|-------------------|
| Macrogametocytes               | n = 26            |
| Length                         | 15.1–26.0 (20.8 ± 3.0) |
| Width                          | 4.6–10.2 (7.1 ± 1.7) |
| Parasite nucleus               |                   |
| Length                         | 3.2–7.0 (5.1 ± 0.9) |
| Width                          | 2.8–4.8 (3.2 ± 0.5) |
| Host-cell nucleus              |                   |
| Length                         | 6.9–14.9 (11.0 ± 2.3) |
| Width                          | 1.6–4.0 (2.5 ± 0.6) |
| Cytoplasmic processes          |                   |
| Length                         | 9.5–21.7 (15.5 ± 3.7) |
| Width                          | 1.8–6.4 (3.8 ± 1.0) |
| Host-cell parasite complex     |                   |
| Length                         | 30.1–61.8 (46.0 ± 9.1) |
| Width                          | 5.6–13.9 (9.6 ± 2.3) |

a Minimum and maximum values are provided, followed in parentheses by the arithmetic mean and standard deviation.

b Cytoplasm of fusiform host cell which forms processes at the ends of gametocyte.

4. Discussion

Leucocytozoids have been recorded from a large number of bird species around the world (Greiner and Kocan, 1977). Diurnal raptors from Accipitridae are highly parasitized by leucocytozoids (Walther et al., 2016). Epidemiological studies have indicated that Leucocytozoon infection in birds from Accipitridae is ten times more common than Falconidae (Valkiunas, 2005).

Out of the 455 common buzzard nestlings which were examined in Germany, 40 % were infected with Leucocytozoon (Chakarov et al., 2008). The prevalence rate of leucocytozoids in raptors from different

regions is more related to the avian species, climatic conditions, distribution of insect vectors, and also the methods applied for diagnosis (Walther et al., 2016; Nourani et al., 2020). There is a tendency for a higher prevalence of the L. toddi group in the direction from the tropical latitudes to the middle latitudes of the Northern Hemisphere (Valkiunas, 2005). Our microscopic results indicate a high prevalence of L. buteonis (54.5 %) in free-living common buzzards. The high prevalence of L. buteonis in apparently healthy adult birds suggests that this is a harmless endemic haemoparasite of common buzzards in west of Iran.

Some avian Haemoproteus spp. create acute illness and intensive mortality through exo-erythrocytic meronts (Valkiunas and Iezhova, 2017). Evidence suggests that the exo-erythrocytic stages might be different in their manifestations in the avian hosts, which could probably be dependent on various factors, including host diversity, new emergences of the parasite, and infection of birds with unspecified strains. Olias et al. (2011) reported a parakeet infected with H. minutus that produced deadly megalomeronts while parasitemia was absent. In this regard, it has been propounded that many haemosporidian strains have different developmental stages in the aberrant hosts. Tissue stages can develop in these hosts, but these structures will be arrested where the erythrocytic stages do not appear (Valkiunas and Iezhova, 2017).

Prior to the availability of molecular and genetic phylogenies, there was little information about the exo-erythrocytic stages of haemoproteids, and the identification was mainly based on morphological characteristics. If genetic lineages of haemosporidian parasites are linked with morphospecies, a remarkable volume of information would be available for phylogenetic analysis of these blood parasites (Valkiunas and Iezhova, 2017). In the present study, exo-erythrocytic stages of the genus Haemoproteus were identified in the histological sections. The cytb gene obtained from tissue samples confirmed the Haemoproteus sp. infection. The studied sequence belongs to an undescribed lineage (BUTBUT15). It was placed in a different clade, separate from other sequences recorded in Buteo buteo, closer to lineages isolated from Falco naumanni and other falcon species. From a phylogenetic standpoint, the species of the studied Haemoproteus sp. is likely to be different from other reports from Buteo buteo and might be a new species. However, it was linked with two known morphospecies (H. braciatus and H. tinnoculii), according to the 5 % genetic distance criterion proposed by Hellgren et al. (2007).
Some *Haemoproteus* spp. lineages have reportedly a low degree of host specificity (Szymanski and Lovette, 2005; Nourani et al., 2018). The lineage BUBIBI01 has been found in different raptors, including buzzards (*Buteo buteo* and *Butastur indicus*), owls (*Bubo bubo*, *Athene noctua*, and *Asio flammeus*), falcons (*Falco* spp.), Eurasian sparrowhawk (*Accipiter nisus*), as well as Cattle egret (*Bubulcus ibis*).

There is evidence that shows the successful transmission of the hemoproteids between the birds (Nourani et al., 2018). Our phylogenetic analysis shows a close relationship between the *Haemoproteus* lineages recorded in birds of prey, in which isolates from *Buteo buteo* are placed adjacent to isolates from other raptors. Previous studies have confirmed the ambiguity of phylogenetic analysis of raptor hemoproteids. Ortego et al. (2007), in the study of *Haemoproteus* spp. in *F. naumanni*, confirmed three morphologically described species as well as other undescribed lineages, identified by unique cytb sequences. All the recovered sequence-based lineages were not specific to Falconiformes. The study of Ishak et al. (2008) revealed a lack of phylogenetic cohesion among raptor blood parasites. In addition, Outlaw and Ricklefs (2009) mentioned the overall lack of phylogenetic relatedness among raptor haemosporidians.

Iran is situated at the crossroads of several major bird migration routes and may be influenced to have a high risk of exposure to new parasites (Nourani et al., 2018). *Falco* spp. (eg, *naumanni* and *subbuteo*) are common summer visitors, and the Eurasian kestrel (*Falco tinnunculus*) is a permanent inhabitant of the west of Iran (Mansoori, 2013). *Falco naumanni* is a trans-Saharan migrant and is likely to be infected by a wide array of avian blood parasites from Asia and Africa (Waldenström et al., 2002). Migratory falcons are likely to act as vehicles for...
introducing new species of *Haemoproteus* to the Iranian population of *Buteo buteo*.

5. Conclusion

The present study provided new achievements based on molecular and histopathological findings of *Haemoproteus* sp. in *Buteo buteo*. Close phylogenetic relationship between the studied *Haemoproteus* sp. and lineages isolated from *Falco naumanni*, other members of *Falco* genus and Cattle egret, which is not a bird of prey, suggest that lineage BUT-BUT15 might be a *Haemoproteus* sp. with a wide range of avian hosts. This study reports the first investigation on exo-erythrocytic stages of *Haemoproteus* sp. in common buzzards. Moreover, this is the first histopathological study of tissue stages of haemosporidians in Iran. Comprehensive and inclusive research is still required to clarify the undefined structural characteristics and pathogenesis of haemosporidian parasites.
Declaration of competing interest

All authors declare that they have no competing interests.

Acknowledgements

This work was financially supported by the Lorestan University. We would like to thank the laboratory staff of the Faculty of Veterinary Medicine for their assistance in collecting and examine the samples.

References

Bensch, S., Stjernman, M., Hasselquist, D., Stman, O., Hansson, B., Westerdahl, H., Turillazzi, S., Vinther, J., 2005. Phylogeography and evolution of passerine birds: twelve million years of diversification. Proc. Roy. Soc. B Biol. Sci. 272, 2069–2078.

Bensch, S., Hellgren, O., Perez-Tris, J., 2009. MalAvi: a public database of malaria parasites and related haemosporidians in avian hosts. Mol. Phylogenet. Evol. 53, 260–262.

Bensch, S., Stjernman, M., Hasselquist, D., Stman, O., Hansson, B., Westerdahl, H., Turillazzi, S., Vinther, J., 2006. The phylogeography of avian malaria: a phylogenetic analysis of mitochondrial cytochrome b lineages from sixty species of passerine birds. Proc. Biol. Sci. 273, 1177–1184.

Bensch, S., Stjernman, M., Hasselquist, D., Stman, O., Hansson, B., Westerdahl, H., Turillazzi, S., Vinther, J., 2005. The phylogeography of avian malaria: a phylogenetic analysis of mitochondrial cytochrome b lineages from sixty species of passerine birds. Proc. Biol. Sci. 273, 1177–1184.

Bensch, S., Stjernman, M., Hasselquist, D., Stman, O., Hansson, B., Westerdahl, H., Turillazzi, S., Vinther, J., 2005. The phylogeography of avian malaria: a phylogenetic analysis of mitochondrial cytochrome b lineages from sixty species of passerine birds. Proc. Biol. Sci. 273, 1177–1184.

Bensch, S., Stjernman, M., Hasselquist, D., Stman, O., Hansson, B., Westerdahl, H., Turillazzi, S., Vinther, J., 2005. The phylogeography of avian malaria: a phylogenetic analysis of mitochondrial cytochrome b lineages from sixty species of passerine birds. Proc. Biol. Sci. 273, 1177–1184.

Bensch, S., Stjernman, M., Hasselquist, D., Stman, O., Hansson, B., Westerdahl, H., Turillazzi, S., Vinther, J., 2005. The phylogeography of avian malaria: a phylogenetic analysis of mitochondrial cytochrome b lineages from sixty species of passerine birds. Proc. Biol. Sci. 273, 1177–1184.

Bensch, S., Stjernman, M., Hasselquist, D., Stman, O., Hansson, B., Westerdahl, H., Turillazzi, S., Vinther, J., 2005. The phylogeography of avian malaria: a phylogenetic analysis of mitochondrial cytochrome b lineages from sixty species of passerine birds. Proc. Biol. Sci. 273, 1177–1184.