Sci Parasitol 17(1-2):1-6, June 2016
ISSN 1582-1366

ORIGINAL RESEARCH ARTICLE

Microscopy and PCR-based detection of *Leucocytozoon* spp. in Iranian birds of prey

Jamshid Razmyar[1]✉, Gholam Reza Razmi[2], Amin Mirzazadeh[3]

1 – Department of Clinical Sciences, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran.

2 – Department of Pathobiology, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran.

3 – Post Graduate Student in Poultry Health and Diseases, Faculty of Veterinary Medicine, University of Shiraz, Shiraz, Iran.

Correspondence: Tel. +985138805639, Fax +985138763852, E-mail jrazmyar@um.ac.ir

Abstract. In recent years, population decline has been detected in several birds of prey. Although climate change and habitat destruction are likely responsible for this, but we cannot ignore the role of pathogens, especially parasites in raptor's population decline. In this study, we used both microscopic and molecular techniques to examine blood samples collected from raptors referred to the rehabilitation stations in Northeastern of Iran for blood parasites. A total of 36 raptors from members of the family *Falconidae* and *Accipitridae* were included. Samples were collected from June to end of November 2012.

The prevalence of *Leucocytozoon* spp. in microscopic and molecular examination was 11% in both methods. Among the different species that were included in this study, the Golden Eagle *(Aquila chrysaetos)*, the common buzzards *(Buteo buteo)* and the eagle owl *(Bubo bubo)* were three species infected with *Leucocytozoon* spp. It is therefore, concluded that *Leucocytozoon* spp. were prevalent among raptors in Iran and both genotypical and phenotypical methods are applicable.

Keywords: *Leucocytozoon* spp.; Iran; PCR; Raptors.

Received 24/05/2015. Accepted 15/01/2016.

Introduction

Leucocytozoon is a genera of avian hemosporidian belonging to the phylum *Apicomplexa*. These parasites were first seen by Danilewsky (1884). The known vectors of *Leucocytozoon* are black flies of the family *Simuliidae* (Martinsen et al., 2008). Infections with *Leucocytozoon* spp. range from subclinical to clinical signs and mortality, being related considerably to strain of parasite, host species, degree of exposure (Bennett et al., 1994). Acutely affected birds possibly show the sign of anorexia, anemia, ataxia, and tachypnea in some avian species, including chickens and turkey (Bennett et al., 1994).

Leucocytozoon caulleryi is responsible for high levels of mortality in chickens in Japan (Morii and Fukuda, 1992). There are few reports of

mortality caused by blood parasites in wild birds. This is certainly due to the fact that it is almost impossible to routinely monitor this kind of bird in the wild (Bennett et al., 1994). Wild birds tend to have subclinical infections with *Leucocytozoon* spp., although clinical disease and death have been reported in juvenile great horned owls *(Bubo virginianus)* with leucocytozoonosis (Hunter et al., 1997) Birds that survive acute infection appear to enter a chronic phase where parasites are undetectable in blood smears until the infection is reactive by stress or hormonal changes (Atkinson and van Riper, 1991).

Detection of *Leucocytozoon* spp. has been based on morphological analysis using the microscopic techniques (Bennett et al., 1994). A major problem here is the fact that, life stages of *Leucocytozoon* spp. parasites are detectable in peripheral blood for a short period which makes it difficult to detect the parasite (Fallis and Desser, 1977; Blanco et al., 1998). Previous studies indicate that, microscopy was significantly less sensitive than polymerase chain reaction-based method as a tool for determining prevalence of hemosporidian infection (Richard et al., 2002; Durrant et al., 2006). Although Valkiunas and coworkers showed that both microscopy and PCR-based detection methods underestimate approximately the same number of the patent infections (Valkiūnas et al., 2008). However PCR-based methods can provide sequence information that allows for the identification of the parasite lineage (Bensch and Åkesson, 2003) which is impossible by using the microscopic method.

The cytochrome *b* fragment is used for detection and identification of *Haemoproteus, Leucocytozoon* and *Plasmodium* lineages (Waldenstrom et al., 2004). In this study, we used both microscopic and PCR methods to investigate parasites in blood samples collected from raptors referred to rehabilitation stations on northeastern of Iran and subsequently provide sequence information.

Materials and methods

The blood samples used in this study were collected from birds of prey hospitalized at rehabilitation stations (N=30). The place that birds brought in for treatment and recovery after being found injured or sick and then prepare them for releasing or captured raptors referred by owners to our veterinary hospital (N=6). Blood samples (2–4 ml) were collected from the brachial wing vein using Potassium EDTA as the anticoagulant, in the following six species (table 1): *Falconidae* and *Accipitridae:* Golden eagle *(Aquila chrysaetos),* Saker falcon *(Falco cherrug),* Kestrel *(Falco tinnunculus),* common buzzard *(Buteo buteo),* harrier *(Pallid harrier)* and eagle owl *(Bubo bubo).* A drop of blood from each sample was immediately smeared, and additional blood was frozen for PCR assay. Samples were collected from June to the end of November 2012.

Table 1. Raptor's species details and microscopic and molecular results

Species	Scientific names	Number of birds examined	Microscopy and PCR positive samples
Golden eagle	*(Aquila chrysaetos)*	7	2(28.5%)
Saker falcon	*(Falco cherrug)*	6	None
Common kestrel	*(Falco tinnunculus)*	4	None
Common buzzard	*(Buteobuteo)*	16	1(6.25%)
Pallid harrier	*(Circus macrourus)*	1	None
Eagle owl	*(Bubo bubo)*	2	1(50%)
Total		**36**	**4(11%)**

Total 36 blood smears were made on-site and air-dried and subsequently fixed in 97% absolute methanol before staining with Giemsa (Godfrey et al., 1987). To examine the parasites morphologically, smears were viewed microscopically at ×200, ×400, and ×1000.
DNA was extracted using the Blood DNA extraction kit according to manufacturer's instructions (Bioneer™, South Korea).

Extracted DNA was used in two sets PCR reactions to amplify a portion of the cytochrome *b* gene using two previously published data with minor modifications (Sehgal et al., 2006).

PCR materials used in reaction were provided by Ampliqon (Odense, Denmark). Amplification reactions were carried out in a 50 µl reaction volume containing 25 µl of the Taq DNA Polymerase 2x Master Mix (two mM MgCl2 final concentration), Tris-HCl pH 8.5, (NH4)2S04, four mM MgCl2, 0.2% Tween 20, 0.4 mM dNTPs 0.2 units/µl AmpliqonTaq DNA polymerase, Inert red dye and stabilizer, 10 µl of template DNA, 1µl of each primers (10 pm/µl) and dH2O up to 50 µl. We used the following primers with 865 bp products as diagnostic PCR and for sequencing: LeucoF:5-TCTTACTGGTGTATTATTAGCAAC-3 and LeucoR:5-AGCATAGAATGTGCAAATAAACC-3.
All the PCRs were performed in duplicate. The cycling profile consisted of denaturation at 94°C for 3 min, followed by 35 cycles of 94°C denaturation for 30 sec, 52°C annealing for 30 sec, and 72°C extension for 1 min. The samples were then extended at 72°C for 10 min (Sehgal et al., 2006). The 865 bp amplification products were detected by gel electrophoresis (Padideh-Nojen Pars, Iran) in 1.5% agarose gel in 1 x TAE buffer, stained with 0.5 µg/ml EtBr. Amplified bands were visualized and photographed under UV transillumination.

Published sequences for the cytochrome *b (cyt b)* genes of *Leucocytozoon* spp.*,* using alignment software, obtained from GenBank. Positive sample (the first positive sample confirmed by sequencing), and negative controls (blank sample) were used as well, according to standard protocols.

The PCR products were then purified using the Bioneer purification kit and were submitted for an automated sequencing in both directions (Macrogen Inc. South Korea) using PCR primers as the sequencing primers. Nucleotide (nt) and predicted amino acid (aa) sequence data were aligned with the Clustal alignment algorithms. Phylogenetic analysis based on the nt sequences was conducted using a distance method, unweighted pair group with arithmetic mean, and by calculating bootstrap values for 1000 replicates in CLC workbench 5.5 (CLC Bio, Denmark). The sequence data was submitted to GenBank under the accession numbers.

Results

Four out of 36 raptors in blood parasite identification were *Leucocytozoon* spp. positive in the microscopic (figure 1) and molecular assay. Positive samples belonged to two Golden eagles *(Aquila chrysaetos)*, one Common buzzards *(Buteo buteo)* and one to Eagle owls *(Bubo bubo),* moreover the were identical in sequencing analysis of nucleotide and deduced amino acide levels. All the negative samples in microscopic examination were negative in PCR assay. Sequences are deposited in GenBank with the following accession number; KF146933-4. The LeucoRRA100 and LeucoRRA101 are located in a main branch, the strains we found are nestled in a clade that found in Leucocytozoon danilewsky with accession number EU627823 (figure 2).

Discussions

A few parasitological surveys have been done on blood parasites among wild birds in Iran. Although the study on raptors is considerably significant, it is just limited to one report of *Leucocytozoon* spp. infection in common Buzzard *(Buteo buteo).* The prevalence of *Leucocytozoon* spp. infection was determined in 5.1% of chickens, 4.3% of geese, 3.6% of ducks and 2.9% of turkeys in southwest of Iran (Dezfoulian et al., 2013). In this study, the frequency of *Leucocytozoon* spp*.* in raptors, was 11%, which had been detected by both microscopy and PCR.

Various studies on the prevalence of *Leucocytozoon* spp. in wild birds have been done in neighboring countries of Iran such as United Arab Emirates and Turkey. Prevalence of *Leucocytozoon* spp. was reported 0.9% in falcons from United Arab Emirates (Lierz et al., 2005) and were observed in only five pigeons and one owl chick out of 53 wild birds of the border district from Turkey (Ozmen et al., 2005). In other studies worldwide, 11% of wild birds in north California were infected with *Leucocytozoon* spp. (Martinsen et al., 2008).

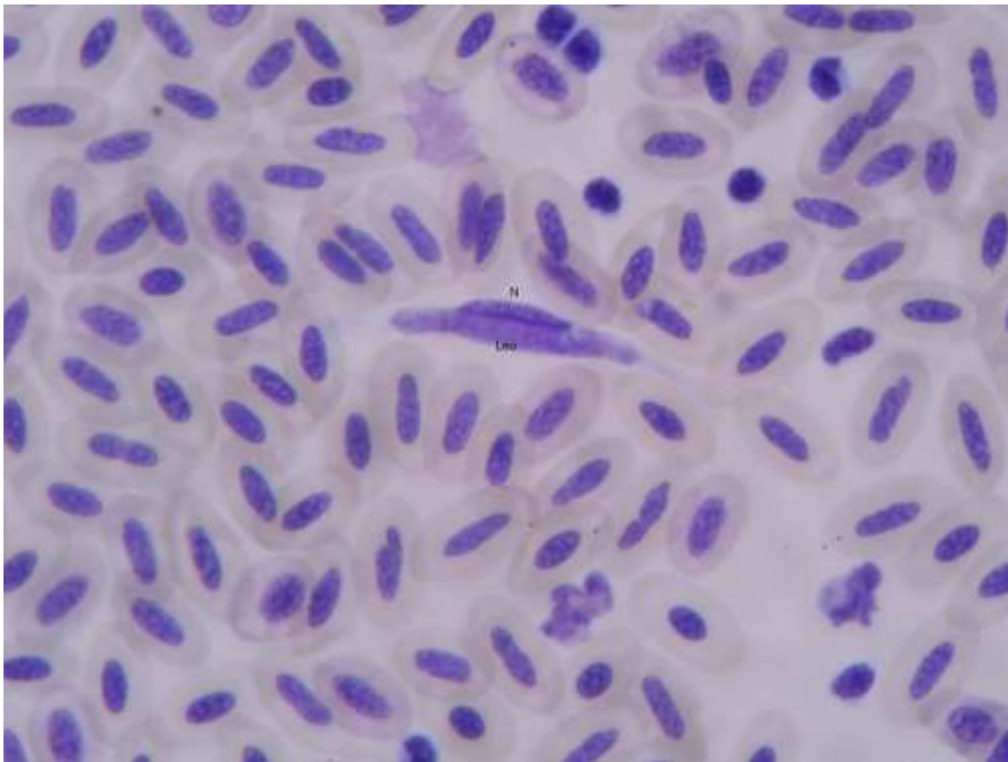

Figure 1. Blood smear; gametocytes of *Leucocytozoon* spp. (leu) located in red or white blood cells. The host cell nucleus (N) is pushed to one pole (Giemsa stain; Magnification 1000×). *Leucocytozoon* parasite from a blood smear of a Common Buzzards *(Buteo buteo).* The sample was taken in 2012.

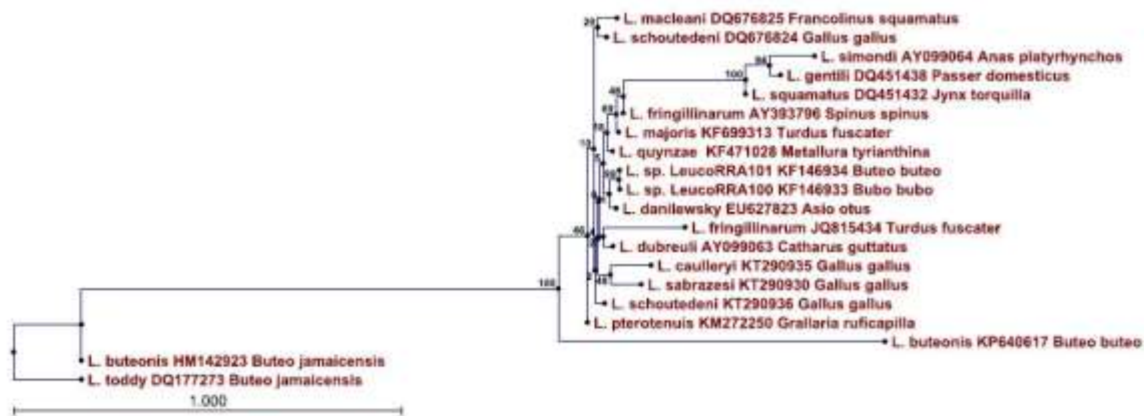

Figure 2. The Phylogenetic tree of selected *Leucocytozoon* spp. cytochrome b gene sequences based on the nucleotide sequence (450 bp); Branched distances correspond to a sequence divergence. The LeucoRRA100 (KF146933) and LeucoRRA101 (KF146934) are located in a main branch, the strains we found are nestled in a clade that found in *Leucocytozoon danilewsky* with accession number EU627823. We used *Leucocytozoon toddi* as an out-group in the phylogeny.

Thirteen of 21 nestling bald eagles in Michigan and Minnesota were *Leucocytozoon* spp. positive (Stuht et al., 1999). The microscopies of blood smears show *Leucocytozoon* spp. infection in 43.2% of raptors from raptors of southern New Jersey and adjacent area (Kirkpatrick and Lauer, 1985) and 29.9% of diurnal raptors from California (Sehgal et al., 2006). Haemo-protozoan parasites were not found in blood smears of 82 Griffon vultures

that examined in Spain (Blanco et al., 1998). In a study from Japan, 4.6% of blood samples in 701 Japanese wild birds were *Leucocytozoon* spp. positive during a period of 13 years (Murata, 2002).

Vector-born disease such as blood parasites reduces wild birds survival and is also related to conservation biology because endangered species may be pushed to extinction (Kilpatrik et al., 2006). If parasites loads increased it can negatively affects host population, it can reduce growth and causing higher mortality and lower birth rate. Furthermore parasites can have detrimental effects on host fitness and cause oxidative stress on breeding stage (Van de Crommenaker et al., 2011) and they can produce heat shock proteins in this condition as well (Martínez-de la Puente et al., 2012).

The result shows that *Leucocytozoon* spp. is prevalent among Iranian birds of prey population and is seems to be responsible for raptor's population decline. The purpose of this investigation was to look for occurrences of blood parasites in Iranian raptors and to begin to assess the impact of blood parasites on Iranian birds of prey population in further works. More research is required to determine the prevalence of *Leucocytozoon* in Iran bird's population, especially in raptors, along with comparative studies assessing ecological condition regulating host and vector relation. Possible infected host birds must be immediately diagnosed for the infection, and isolated *Leucocytozoon* should be classified to the species level for effective veterinary control.

Acknowledgments

The authors would like to thank Mr. Ali Kargar for his valuable technical help and notify that the research was funded by the grant No. 24403 from the Research Council of the Ferdowsi University of Mashhad.

References

Atkinson C.T., van Riper C.III. 1991. Pathogenicity and epizootology of avian haematozoa: *Plasmodium*, *Leucocytozoon*, and *Haemoproteus*. *In:* Loye J.E., Zuk M. (Eds.), Bird-parasite interactions. Oxford: Oxford University Press. pp. 19-48.

Bennett G.F., Peirce M.A., Earlé R.A. 1994. An annotated checklist of the valid avian species of *Haemoproteus, Leucocytozoon* (Apicomplexa: Haemosporida) and *Hepatozoon* (Apicomplexa: Haemogregarinidae). Syst. Parasitol. 29:61-73.

Bensch S., Åkesson S. 2003. Temporal and spatial variation of hematozoans in Scandinavian willow warblers. J. Parasitol. 89:388-391.

Blanco G., Gajón A., Doval G., Martínez F. 1998. Absence of blood parasites in Griffon vultures from Spain. J. Wildlife Dis. 34:640-643.

Dezfoulian O., Zibaei M., Nayebzadeh H., Haghgoo M., Emami-Razavi A.N., Kiani K. 2013. Leucocytozoonosis in domestic birds in Southwestern Iran: an ultrastructural study. Iran. J. Parasitol. 8:171-176.

Durrant K.L., Beadell J.S., Ishtiaq F., Graves G.R., Olson S.L., Gering F., Perice M.A., Milensky C.M., Schmith B.K., Gebhard C., Fleischer R.C. 2006. Avian haematozoa in South America: A comparison of temperate and tropical zones. Ornithological Monographs 60:98-111.

Fallis A.M., Desser S.S. 1977. On species of *Leucocytozoon, Haemoproteus,* and *Hepatocystis. In:* Kreier J.P. (Ed.), Parasitic Protozoa. Academic Press, New York, p. 239-266.

Godfrey R.D., Fedynich A.M., Pence D.B. 1987. Quantification of hematozoa in blood smears. J. Wildlife Dis. 23:558-565.

Hunter D.B., Rohner C., Currie D.C. 1997. Mortality in fledgling great horned owls from black fly hematophaga and leucocytozoonosis. J. Wildl. Dis. 33:486-491.

Kilpatrick A.M., LaPointe D.A., Atkinson C.T., Woodworth B.L., Lease J.K., Reiter M.E., Gross K. 2006. Effects of chronic avian malaria *(Plasmodium relictum*) infection on reproductive success of Hawaii Amakihi *(Hemignathus virens).* Auk 123:764-774.

Kirkpatrick C.E., Lauer D.M. 1985. Hematozoa of raptors from southern New Jersey and adjacent areas. J. Wildlife Dis. 21:1-6.

Lierz M., Hafez H.M., Krone O. 2005. Prevalence of hematozoa in falcons in the United Arab Emirates with respect to the origin of falcon hosts. J. Avian Med. Surg. 22:208-212.

Martínez-de la Puente J., Merino S., Tomás G., Moreno J., Morales J., Lobato E., Martínez J. 2012. Nest ectoparasites increase physiological stress in breeding birds: an experiment. Naturwissenschaften 98:99-106.

Martinsen E.S., Blumberg B.J., Eisen R.J., Schall J.J. 2008. Hemosporidian parasites from northern California oak woodland and chaparral habitats. J. Wildlife Dis. 44:260-268.

Morii T., Fukuda M. 1992. Observations on first-generation schizogony of *Leucocytozoon caulleryi* in chickens. J. Protozool. 39:281-287.

Murata K. 2002. Prevalence of blood parasites in Japanese wild birds. J. Vet. Med. Sci. 64:785-790.

Ozmen O., Halıgür M., Yukarı B.A. 2005. A study on the presence of leucocytozoonosis in wild birds of Burdur. Turk J. Vet. Anim. Sci. 29:1273-1278.

Sehgal R.N., Hull A.C., Anderson N.L., Valkiŭnas G., Markovets M.J., Kawamura S., Tell L.A. 2006. Evidence for cryptic speciation of *Leucocytozoon* spp. (Haemosporida, Leucocytozoidae) in diurnal raptors. J. Parasitol. 92:375-379.

Stuht J.N.W., Bowerman W., Best D.A. 1991. Leucocytozoonosis in nestling bald eagles in Michigan and Minnesota. J. Wildlife Dis. 35:608-612.

Valkiunas G., Iezhova T.A., Krizanauskiene A., Palinauskas V., Sehgal R.N.M., Bensch S. 2008. A comparative analysis of microscopy and PCR-based detection methods for blood parasites. J. Parasitol. 94:1395-1401.

Van de Crommenacker J.D., Richardson S., Koltz A.M., Hutchings K., Komdeur J. 2011. Parasitic infection and oxidative status are associated and vary with breeding activity in the Seychelles warbler. P. Roy. Soc. B-Biol. Sci. 279:1466-1476.

Waldenström J.S., Bensch S., Hasselquist B.Q., Östman O. 2004. A new nested polymerase chain reaction method very efficient in detecting *Plasmodium* and *Haemoproteus* infections from avian blood. J. Parasitol. 90:191-194.