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Detection of Haemosporidia in Healthy Pet Parrots in South Korea

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Abstract : Avian haemosporidia, including malarial parasites, are geologically and biologically widespread. The protozoal pathogen has been a subject of intensive research in the past, which has resulted in major medical progress. Haemosporidia infection in avian species in South Korea has been studied in wild birds and layer flocks, but not in pet birds. At the Veterinary Teaching Hospital of Chungbuk National University, 75 birds that presented for health check-up were tested to evaluate the infection rate of *Haemoproteus*, *Plasmodium* and *Leucocytozoon* in birds without clinical symptoms. *Haemoproteus* spp. and *Leucocytozoon* spp. were simultaneously detected in a Major Mitchell's cockatoo (*Lophochroa leadbeateri*) by polymerase chain reaction, representing 1.33% of the tested birds. Phylogenetic analysis suggested that the infective *Haemoproteus* and *Leucocytozoon* strains were similar to those detected in foreign countries rather than those detected in the wild birds of Korea. Although the infection rate may not be indicative of a substantial infection in healthy pet parrots, the import of infected birds can pose a threat by allowing foreign pathogens to infect the local wild flocks or livestock. This is the first surveillance study of avian haemosporidia in pet parrots in South Korea.

Key words **:** avian malaria, haemosporidia, *Haemoproteus*, *Leucocytozoon*, parrots.

Introduction

Psittacines as well as other avian species form a growing field of research in veterinary medical services in South Korea. Avian cases at the Chungbuk National University Veterinary Teaching Hospital (CBNUVTH) increased from 107 cases in 2016 to 322 cases in 2019, almost tripling in number. As the interest in the avian species is growing, the need to understand the nature of their infectious diseases is also gaining attention. Tests for psittacine beak and feather disease, avian polyoma virus, and psittacosis and avian bornavirus (Proventricular dilatation disease) are most frequently requested when adopting new psittacine members in South Korea. Testing for these diseases is generally recommended as a routine procedure to adopt an avian family member in South Korea.

In the past, avian haemosporidia have served as an important disease model for research on parasite-host interactions (15,17), chemical therapy (3), and vaccine development (18) for malaria in humans, thus reinforcing our understanding and control over the disease. Although recent studies employ rodents instead of avians for the disease model, numerous studies conducted in the past have built a substantial database, making avian haemosporidia a significant model for the study of other parasitic diseases (19).

Although there are not as many reports regarding avian haemosporidia in South Korea as compared to the neighboring countries, the temperate climate supports infestation of at least 16 species of mosquitoes every summer (11). These vectors are a significant factor in the spread of haemosporidian diseases. Furthermore, it has been reported that an alarming proportion of almost 45% of mature wild birds in South Korea are constantly infected (15). Hence, it is essential to investigate the current prevalence of haemosporidia in indoor birds to improve disease prevention among companion birds.

With the recent recurrence of various zoonotic diseases, the importance of health has been emphasized more than ever. The popularity of avian species as companion animals is growing, with a simultaneous increase in the import of foreign exotic birds. This could also lead to unintentional introduction of foreign pathogens such as parasites into new regions, with the imported animals acting as carriers. Analyzing the prevalence and distribution will allow the evaluation of the potential threat of avian haemosporidia in companion animals and local flora in South Korea.

Materials and Methods

Sample collection

Frozen DNA extractions of blood samples were tested retrospectively. In this study, psittacines presented to the CBNUVTH for health examinations were selected to determine the infection prevalence among clinically asymptomatic psittacine pet birds. A total of 75 birds, examined between January 2019 and June 2020, were included. Blood samples were collected from the right jugular vein of each bird.

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Table 1. Primers for avian haemosporidia amplification

Detection	Primer*	Sequence
Nest	HaemF	5′-CATATATTAAGAGAAITATGGAG-3′
	HaemR	5′-ATAGAAAGATAAGAAATACCATTC-3′
Haemoproteus spp. *Plasmodium* spp.	HaemF2	5′-ATGGTGCTTTCGATATATGCATG-3′
	HaemR2	5′-GCATTATCTGGATGTGATAATGGT-3′
Leucocytozoon spp.	Leuco-HaemFL	5′-ATGGTGTTTTAGATACTIACATT-3′
	Leuco-HaemR2L	5′-CATTATCTGGATGAGATAATGGIGC-3′

*Hellgren et al. (7).

DNA extraction

DNA was extracted from the samples using Magipurix® Blood NAs B kit and Magipurix® 12s automated nucleic acid purification system (Zinexts Life Science Corp., New Taipei City, Taiwan), according to the manufacturer's protocol. Samples were extracted for DNA upon collection and stored at −20°C until the polymerase chain reaction (PCR) amplification.

Polymerase chain reaction

Nested PCR was selected as the amplification method to ensure sensitive detection as described by Hellgren et al. (8). Primers HaemF and HaemR were used for the first amplification. The second amplification was conducted separately for *Haemoproteus*/*Plasmodium* and *Leucocytozoon*. *Haemoproteus* and *Plasmodium* were amplified using HaemF2 and HaemR2 in the second amplification, whereas *Leucocytozoon* was amplified using Leuco-HaemFL and Leuco-HaemR2L (Table 1). The first amplification was performed using a mixture of 1.75 μL of the extracted DNA template, 5 μL PCR buffer, 5 μL deoxynucleoside triphosphate (dNTP), 2 μL of each primer, 1.5 μL magnesium chloride, 0.5 μL bovine serum albumin, 0.25 μL Taq polymerase, and 32 μL water. The mixture was denatured for 5 min at 95°C, followed by 40 cycles at 95°C for 30 s, 50°C for 45 s, and 72°C for 60 s, and finally extended at 72°C for 10 min before being held at 4°C. The second amplification mixture comprised of 1.75 μL of primary amplified template, 33.25 μL water, 5 μL PCR buffer, 5 μL dNTP, 5 μL Taq mix, 2 μL of each primer, 0.75 μL magnesium chloride, and 0.25 μL Taq polymerase. The second amplifications for *Haemoproteus* and *Plasmodium* were initially denatured at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 56°C for 35 s,

Table 2. PCR-positive rates for avian haemosporidia in different species of psittacine birds

Genus	Species	Positive/Total
Agapornis	*Roseicollis*	0/6
Amazona	*Aestiva*	0/6
Amazona	*Ochrocephala*	0/10
Ara	*Ararauna*	0/2
Ara	*Macao*	0/3
Aratinga	*Solstitialis*	0/3
Bolborhynchus	*Lineola*	0/1
Cacatua	*Galerita*	0/1
Diopsittaca	*Nobilis*	0/3
Eclectus	*Roratus*	0/1
Eolophus	*Roseicapilla*	0/1
Lorius	*Lory*	0/1
Lophochroa	*Leadbeateri*	**1/3**
Myiopsitta	*Monachus*	0/7
Nymphicus	*Hollandicus*	0/1
Pionites	*Melanocephalus*	0/1
Poicephalus	*Cryptoxanthus*	0/1
Poicephalus	*Gulielmi*	0/1
Poicephalus	*meyeri*	0/1
Poicephalus	*Senegalus*	0/4
Psittacula	*Krameria*	0/1
Psittacus	*Erithacus*	0/9
Pyrrhura	*Molinae*	0/8
Total		1/75*

*Overall positive rate is 1.33%.

Table 3. Point mutations in the cytochrome *b* region of amplified strains

Reference: KU160476 (*Haemoproteus minchini*)	**Reference: AB741500** (*Leucocytozoon* spp.)
m.10 C > T	m.175 A > T
m.34 T > C	m.493 T > C
m.46 C > T	
m.55 A > C	
m.65 T > C	
m.104 A > T	
m.109 A > T	
m.115 A > T	
m.122 T > C	
m.127 T > C	
m.214 T > C	
m.283 T > C	
m.295 T > C	
m.352 T > C	
m.388 A > G	
m.431 G > A	
m.452 T > C	
m.454 A > T	
m.484 A > T	
19 mutations	2 mutations

and extension at 72°C for 50 s, followed by a final extension at 72°C for 7 min before holding at 4°C. The exact same denaturation and cycles were performed for *Leucocytozoon* separately, except for annealing performed at 50°C for 35 s. All PCR products were separated on 1.5% agarose gel and were visualized using an ultraviolet lamp.

DNA sequencing

The PCR amplicons were sequenced bidirectionally. Sequencing reactions were performed using BigDye Terminator Chemistry (Version 3.1) Cycle Sequencing Kit (Thermo Fisher Scientific, Waltham, USA) according to the manufacturer's instructions. PCR amplification was performed for a total volume of 10 μL with 10 ng of the template, 5 pmole/μL of each primer (HaemF2 and HaemR2 for the *Haemoproteus* template; Leuco-HaemFL and Leuco-HaemR2L for the *Leucocytozoon* template) (Table 1), and 0.5 μL of Terminator Ready Reaction Mix. Following this, up to 10 μL of distilled water was added using a Verti™ 96-well Thermal Cycler (Thermo Fisher Scientific, Waltham, USA). The reaction was performed with initial denaturation at 96°C for 1 min, followed by 30 cycles of denaturation at 96°C for 10 s, annealing at 52°C for 5 s, and extension at 60°C for 4 min then held at 10°C. The PCR products were then resolved using an ABI 3730XL DNA analyzer (Thermo Fisher Scientific, Waltham, USA).

Phylogenetic analysis

Sequence information from amplified PCR products was assembled and analyzed using CLC Main Workbench 20 (CLC Bio, Aarhus, Denmark) and then aligned to reference sequences obtained from GenBank. Reference sequences included sequences with close resemblance to the strain from this study reported from various geological backgrounds, as well as from geologically intimate countries in northeast Asia, although not as closely related, and sequences from wild birds in South Korea. A phylogenetic tree was created using the Kimura 80 method, with bootstrap values based on 100 replicates.

Results

Prevalence

This study examined 75 cases, which consisted of 23 dif-

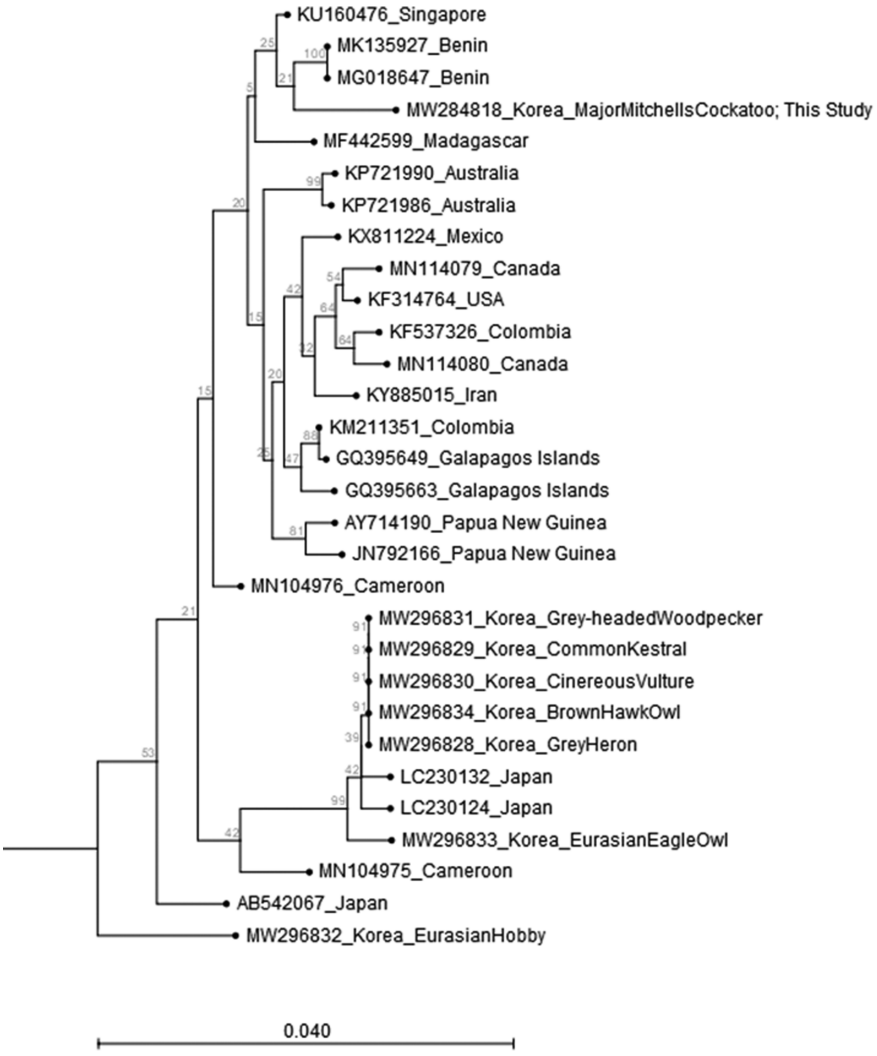

Fig 1. Haemoproteus strain phylogenetic tree. Sequences obtained from Genbank were included. Phylogenetic analysis was performed using Kimura 80 method based on 100 replicates.

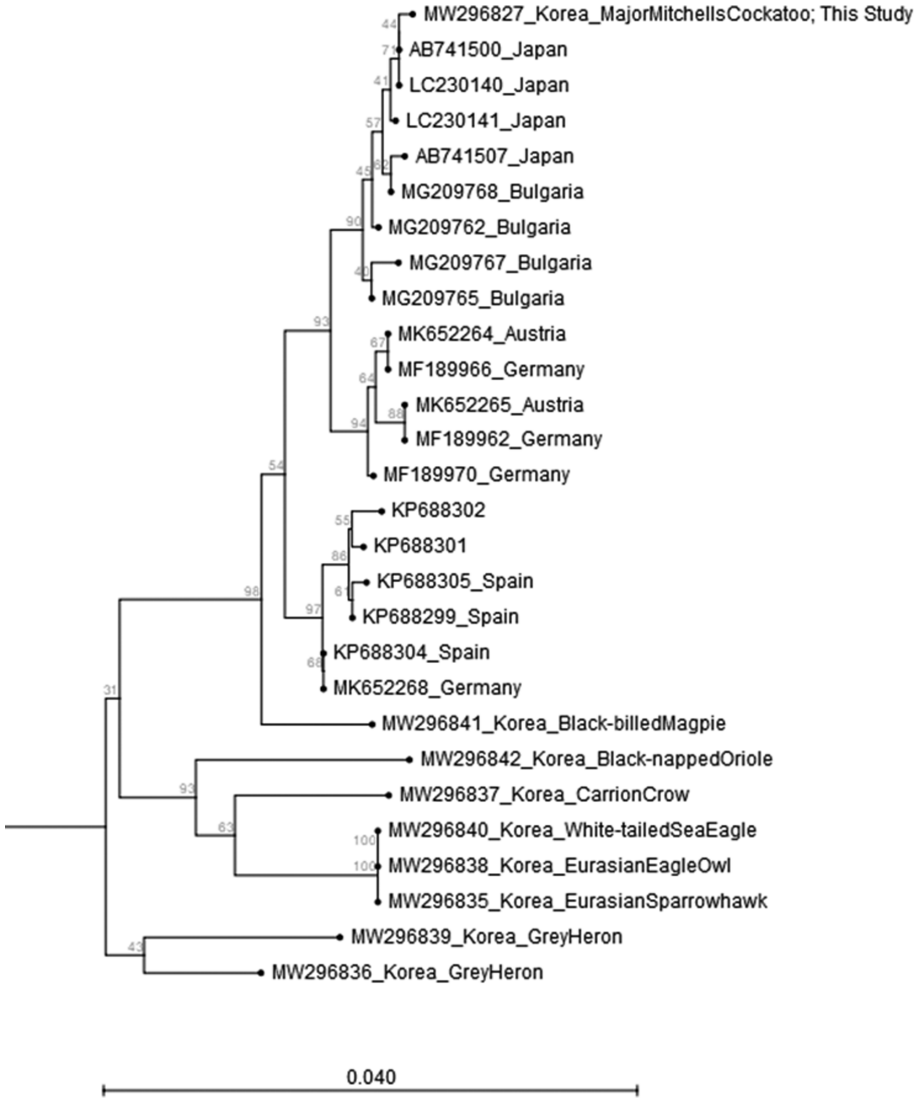

Fig 2. Leucocytozoon strain phylogenetic tree. Sequences obtained from Genbank were included. Phylogenetic analysis was performed using Kimura 80 method based on 100 replicates.

ferent species of exotic companion birds (Table 2). Only a single sample tested positive, with a co-infection of *Haemoproteus* and *Leucocytozoon*. Due to the low prevalence rate, other factors such as sex predisposition or seasonal differences were not eligible for analysis. The positive sample was obtained from a Major Mitchell's cockatoo (*Lophochroa leadbeateri*), a mid-sized psittacine native to Australia. It was presented to the CBNUVTH for sex identification. No clinical symptoms were observed upon presentation.

DNA sequencing

The amplified cytochrome *b* DNA segments were compared to a reference strain retrieved from GenBank (Table 3). The closest resemblance reported in GenBank to the infective *Haemoproteus* spp. strain of this case was isolated from a flock of Great Blue Turacos (*Corythaeola cristata*) in a bird park located in Singapore (5). When aligned, the strain from this study had 19 modified nucleotides, with 96% identity between the two strains. On the other hand, the *Leucocytozoon* spp. strain of this case had two modified nucleotides when aligned with the *Leucocytozoon* strain isolated from a wild large-billed crow (*Corvus macrorhynchos*) from Japan (9) with 99% identity.

Phylogenetic analysis

The strains of the pathogens in this infected case were compared to those registered in GenBank. The *Haemoproteus* strain was closely related to the strains isolated from Singapore (*H. minchini*, GenBank accession number KU160476) and Benin (MK135927 and MG018647), although clearly separated with a high bootstrap value of 100 (Fig 1). The *Leucocytozoon* strain of this case was closely related to the strains isolated from Japan (LC230140 and AB741500), separated by a mid bootstrap value of 46 (Fig 2).

Discussion

Haemosporidia, including *Haemoproteus*, *Plasmodium*, and *Leucocytozoon*, hold certain practical importance in avian species, as these infections could potentially threaten the pro-

ductivity and increase the mortality of production animals or exotic collections. They can reportedly cause anemia, anorexia, and ataxia in infected avians, although the infections may be asymptomatic. Fatty liver, splenomegaly, and regressive reproductive organs, among other gross lesions, are commonly found upon necropsy. This can not only lead to severe loss of production value in industry animals (12), but also cause mass deaths as reported in penguins and psittaciform birds in the zoos of North America and Eurasia (2,7).

Haemosporidia have been previously detected in wild birds and production birds in South Korea. A previous study reported that 45% of wild birds submitted to a wildlife rehabilitation facility tested positive for strains of *Haemoproteus* spp. and *Plasmodium* spp. by PCR (16). Furthermore, a large proportion of infected wild birds belonged to the Strigidae family. Other studies detecting haemosporidia in the Strigidae family may indicate that both wild and captive owls are prone to infections (10,13). *Leucocytozoon* has also been reported in poultry across a few farms in South Korea (12), suggesting the parasitic pathogen's ability to infiltrate artificial husbandry.

The positive sample was from a Major Mitchell's Cockatoo (*Lophochroa leadbeateri*) which did not present any clinical symptoms. The *Haemoproteus* strain in GenBank with the highest identity to the strain identified in this study was accession number KU160476. *H. minchini*, first described in 1910 by Minchin and later taxed as a separate species in 2017, reportedly infected captive birds in a bird park in Singapore (5). Other similar strains, KY721990 and KY721986, were detected in Australia, where the Major Mitchell's cockatoo is a native bird (4). The closest related strain of *Leucocytozoon* was isolated from a *C. macrorhynchos* in Japan (9).

Phylogenetic analysis suggests the strains do not resemble strains found in Korean wild birds. Migratory birds have already been suggested as a significant factor in the spread of haemosporidia (14,20). However, the *Haemoproteus* strain detected in this study was closely related to a strain isolated from a faraway region, indicating artificial relocation as another factor of geographical spread.

Vector insects, including *Culicoides arakawae*, are commonly found throughout Asia and can act as a vessel of overseas transfer via human-induced transport or natural phenomena such as typhoons or other strong winds. Studies have also suggested that temperature anomalies resulting from climate change have increased the spread of these pathogens, which may ease the process of pathogens acclimating to the foreign flora (6).

Conclusion

The prevalence rate in this study is relatively insignificant; hence, bird caregivers and veterinarians could be discouraged from testing for haemosporidian infections in exotic birds. However, this should not be the conclusion. Haemosporidians show variable specificity in avian hosts (1), which indicates that pathogens in South Korea have the potential to infect exotic birds imported from foreign countries. On the other hand, foreign strains of pathogens may cause unpredictable pathogenesis when infecting novel hosts. Newly introduced pathogens can lead to unstable pathogenetic relationships between hosts and parasites, resulting in severe epizooties (19). Therefore, it is important that haemosporidia infections are not overlooked in the veterinary care of exotic birds. Future studies should include additional surveillance to investigate clinically sick birds.

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