Original Article

Detection of Malaria Parasites and Other Haemosporidia in Migratory and Native Birds in Mazandaran and Golestan Provinces, Iran

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Abstract

Background: A variety of haemoprotozoa including *Plasmodium*, *Haemoproteus* and *Leucocytozoon* cause infections in birds and are transmitted by some known vectors. These parasites cause anemia, low appetite, weakness and ultimately death in birds. The present study was aimed to determine these parasites, in birds of Mazandaran and Golestan provinces in Iran.

Methods: The project was performed on 340 live birds in 2016. The samples were collected from February to September 2016, from each bird, two thin and thick blood smears were prepared and the remaining blood about 1ml was kept in EDTA-containing tubes for molecular studies. The slides were stained with 10% Giemsa, then examined microscopically. About ten percent of the negative samples were considered for Polymerase Chain Reaction (PCR) technique, using specific primers to diagnose *Plasmodium* and *Haemoproteus* spp. Electrophoresis was done for PCR products and relevant bands to the parasites were identified based on the size. The considered birds belonged to ducks, chickens, roosters, and pigeons.

Results: From 340 microscopically examined blood samples 32 (9.5%) samples were positive. Twenty-five (7.35%) of them were infected with the genus *Haemoproteus*. Seven samples (14%) out of 50 microscopically negative samples were found as *Haemoproteus* or *Plasmodium* spp when PCR technique was employed.

Conclusion: This study revealed the existence of malaria parasites and other haemosporidia in birds in Iran. Employing molecular methods (PCR examination) could detect more infections.

Keywords: Avian malaria parasites; Microscopy; PCR technique; Iran

Introduction

Apicomplexa is a wide range of protozoa which contains many agents with the ability of to produce important diseases. Avian malaria is a parasitic disease of birds caused by species of *Plasmodium* and *Haemoproteus*. These parasites belong to haemosporidia which contains more than 200 species of avian parasites and these have placed into three genera including *Plasmodium*, *Haemoproteus* and *Leucocytozoon* (1). Avian malaria is transmitted by some mosquitoes. Incidence of the disease depends on some factors such as an abundance of vectors, temperature, weather, quality of water and breeding place (2). Species of *Plasmodium* and *Haemoproteus* are pathogens and life-threatening agents for some groups of

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birds particularly those are kept in one place and those are living in warm and humid regions (3). *Plasmodium* and *Haemoproteus* parasites are intercellular agents and growing in red blood cells. These parasites affect birds' Red blood cells (RBCs) in a way that the birds will first suffer anemia and then weakness, lethargy, and distress followed by low appetite, reduced feeding, and consequently death (4).

Vectors of avian malaria can transmit the parasites to the blood circulation of animals even human but rarely results in infection. Moreover, non- infected vectors can pick up the circulating sporozoite from human blood in feeding time (5). Parasites of haemosporina are responsible for millions of infections and thousands of deaths in human, wild and domestic animals. If vectors of these parasites share blood feeding between human and animals, the human community is exposed to a wide range of parasites existing in the animal community (3). Some authors believe that a specific host of a parasite can be modified by natural selection. That is, parasites can naturally adapt themselves to new hosts such as human. It is important to know which kind of animal parasites may be transmitted to human even if these parasites cannot evolve in human in the current situation (5). Indeed, zoonosis may start its primary process based on such transmission form which will adapt in new host gradually. Since these parasites are in close contact with human societies identifying them can help individuals to protect themselves against probable infections.

Over the past five years, few studies have been performed about identifying birds' parasitic infections in the northern part of Iran. The results imply that there is a wide range of parasitic infection among birds. Therefore, it was necessary to renew the relevant study about avian Malaria in the north of Iran regarding the broad spectrum of carriers, vectors, a specific climate of the region, frequent rainfalls, changes in the level of hygiene, especially in rural areas and population growth in this region. On the other hand, besides the health issues portion of the economic life of the native people in the studied areas depends on breeding and selling the birds, so such studies can give some informative hints to the relevant policymakers.

Materials and Methods

Study areas

Samples were collected from Mazandaran and Golestan Provinces from February to September 2016. Mazandaran Province has stretched from the latitude of 35° 47' to 36° 35'N and the longitude of 50° 47'E. The weather in Mazandaran Province is mild with an average temperature of 25 °C in summer and 8 °C in winter. The average annual rainfall in Mazandaran is 700mm. Golestan Province has extended from the latitude of 36° 30' to 38° 8'N and the longitude of 53° 57' to 56° 22'E with an average temperature of 30 in summer and 7 in winter. The average annual rainfall in Golestan is 450mm. Golestan Province has a variety of climates.

Sampling and Testing

A total of 244 and 96 samples were collected from different districts of Mazandaran Province (including Babol, Fereidounkenar and Sari) and Golestan Province (including Gorgan, Gonbad, and Kordkuy).

Domestic birds and wild carnivorous birds were the target groups for this study. The legal guardian was approved from Tehran University of Medical Sciences. The birds were captured randomly using traditionally way including provide plenty of food and water in trap cages, then two milliliters whole blood were collected of their wings and put into EDTA containing tubes via venipuncture to prepare thin and thick blood smears and designing molecular tests as well. Finally the birds were released.

Thin and thick smears were air-dried and stained with 10% Giemsa stain for 20 minutes,

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then the slides were washed up smoothly and after drying assessed under an optical microscope (Leitz, Germany), with 1000 x magnification.

Molecular Analyses

In this study, 50 blood samples including all the suspected samples and 40 of negative samples were examined using the polymerase chain reaction (PCR) test. DNA was extracted from the blood samples using a DNA extraction kit (Bioneer, Cat No: k-3032). As RBCs of birds contain a nucleus and thus a large amount of DNA, so the blood samples were kept at 95 °C for 15 minutes as an additional step to obtain favorable results. To identify the *Plasmodium* genus, a set of primer (PLS and UNR primers as the following) were used to amplify the small subunit ribosomal DNA gene. Development of a 787bp band revealed birds' infection with *Plasmodium* (6). The (P1) and (P2) primers were used to amplify the cytochrome b gene where the formation of a 500bp band showed the birds' infection with *Haemoproteus* (7). Nucleotide sequences of the primers used in this study are shown in Table 1.

To identify the genus *Haemoproteus* a total volume of 25µl comprising 10µl of master mix [including dNTPs, $MgCl_2$, 10x buffer, *Taq* polymerase], 3µl of DNA extract (50ng), 1µl of each (P1) and (P2) primers in concentration l0pmol and distilled water (D.W) up to 25µl were prepared. To detect the *Plasmodium* genus the needed materials were as the same to *Haemoproteus* identification except replacing primers with PLS and UNR primers. After a brief centrifuge, tubes were transferred to a thermocycler (Thermofisher, USA). The program used for the PCR test to determine *Haemoproteus* was as follows: initial denaturation at 94 °C for 3 minutes, then 35 cycles including denaturation at 94 °C for 1 minute, annealing at 55 °C for 45 seconds, extension at 72 °C for 45 seconds, and eventually extension at 72 °C for 10 minutes. PCR program to detect *Plasmodium* genus was run as follows: initial denaturation 4 minutes at 94 °C then 35 cycles of: denaturation at 94 °C for 45 seconds, annealing at 50 °C for 90 seconds, extension at 72 °C for 2 minutes and finally, extension at 72 °C in 8 minutes. Positive and negative controls were used to compare with samples. A positive and negative control came from samples which was clearly positive and negative in microscopic exams respectively. The products of PCR were put under the electrophoresis process with 1% agarose gel to observe bands corresponding to amplification of specific gene to diagnose parasites genus. Gel agarose was prepared using TAE buffer, including Tris base 242g, Acid acetic glacial 57.1 ml, EDTA 100ml (0.5m, pH: 8) up to 50 times concentration. The utilized products were photographed with a UV illuminator. SPSS software was used for analyzing the results of microscopic examination and molecular tests.

Results

The captured bird in this study belonged to ducks (*Anas platyrhynchos*), chickens (*Gallus gallus domesticus*), roosters (*Gallus Domesticus*), pigeons groups (*Columba livia*) and wild carnivorous birds including Egyptian vultures (*Neophron percnopterus*), Kite bird (*Milvus migrans*), Western Marsh Harrier (*Circus aeruginosus*), common buzzard (*Buteo buteo*), and eagles (*Accipitridae*).

Microscopical examination showed that 23 of 244 blood samples (9.42%) that were collected from Mazandaran Province and two samples (2.08%) of those from Golestan Province were infected as *Haemoproteus* spp. All these infections were detected from the 23 (35.38%) and 25(30.12%) pigeons in Mazandaran and Golestan Provinces, respectively (Fig. 1 and Table 2). None of those examined samples showed *Plasmodium* infection using microscopy method.

To study molecular investigation a total of fifty samples including microscopically suspicious and ten percent of negative blood sam-

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ples were assessed by PCR technique. Gel electrophoresis of PCR product of *Haemoproteus* Cytochrome b gene showed two (4%) *Haemoproteus* spp infected cases in pigeons with a band of 500bp. Moreover, gel electrophoresis of PCR products showed five (10%) cases of *Plasmodium* infection in ducks with a band of 787bp (Figs. 2, 3 and Table 3).

Employing the SPSS software confirmed that significant differences were observed between the microscopic method and PCR technique, df= 49 and P< 0.05.

Table 1. Nucleotide sequences of the primers used in this study

Genus	Gene	Length of PCR (bp)	Oligonucleotides 5'-3'	Reference
Plasmodium	small subunit ribosomal DNA	787	PLS: *AGTGTGTATCAATCGAGTTTC* UNR: *GACGGTATCTGATCGTCTTC*	(6)
Haemoproteus	cytochrome b	500	P1: *ATGGTGCTTTCGATATATGCATG* P2: *CATTATCTGGATGTGATGTGATAATG*	(7)

Table 2. Number of collected blood samples from Mazandaran and Golestan Provinces including type of birds and parasitic infection using microscopic examination

Type of bird	Number	*Plasmodium* spp	*Haemoproteus* spp.
Mazandaran Province			
Ducks	87	0	0
Pigeons	65	0	23(35.38%)
Chickens and roosters	92	0	0
Total	244	0	23 (9.42%)
Golestan Province			
Ducks	41	0	0
Pigeons	18	0	2 (11.11%)
Chickens and roosters	17	0	0
Wild (carnivorous) birds	20	0	0
Total	96	0	2 (2.08%)

Fig. 1. Sampling sites (section of Iran): Mazandaran and Golestan Provinces. Stars show collection sites

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Table 3. Number of *Plasmodium* spp and *Haemoproteus* spp in 50 suspicious and negative blood samples among the studied birds in Mazandaran and Golestan provinces using PCR tests

Type of bird	Number	*Plasmodium* spp	*Haemoproteus* spp
Ducks	15	5 (33.33%)	0
Pigeons	10	0	2 (20%)
Chickens and roosters	11	0	0
Wild (carnivorous) birds	14	0	0
Total	50	5 (10%)	2 (4%)

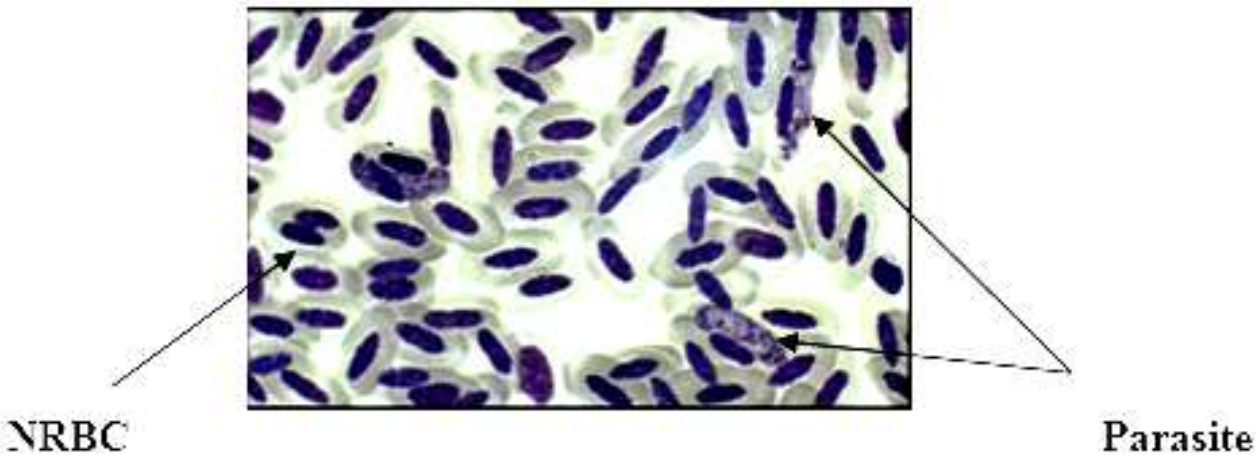

Fig. 2. Morphology of *Haemoproteus* spp in thin blood smear of pigeon based on microscopic method with 1000x magnification. NRBC: nucleated red blood cell, (original picture)

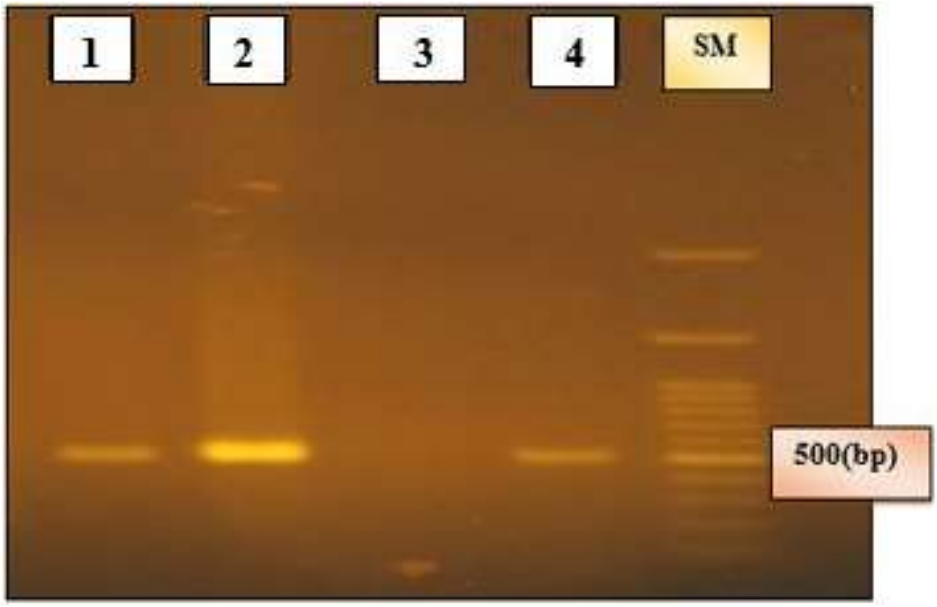

Fig. 3. Gel electrophoresis of the PCR product of *Haemoproteus* Cytochrome b gene, 1 and 2: Positive samples of pigeons, No. 3: Negative control, No. 4: Positive control, SM: Size marker. A 500bp band related to *Haemoproteus* infection is shown in pigeon birds in comparison of positive control

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Fig. 4. Gel electrophoresis of the PCR product of *Plasmodium* small subunit ribosomal DNA gene, No. 1, 2, 3, 4 and 5: Positive samples of ducks. No. 6: Negative control, No. 7: Positive control, SM: Size marker. A 787bp band corresponding to *Plasmodium* infection is shown in duck birds in comparison of the positive control

Discussions

Bird's malaria parasites are transmitted mostly by Culicine mosquitoes that some species of them bite a wide range of hosts including human. Therefore, a human can be exposed to some transmitted parasites through the biting processes. A large number of birds migrate over the winter from Siberia to the province of Mazandaran, and if they carry some species of malaria parasites the disease can be spread over the native birds that are in close contact with the human community, moreover avian have a crucial role in economic life of native residents in this area.

There are a lot of studies about avian malaria and other haemosporidia in the world. According to these revisions the prevalence rate of *Haemoproteus* and *Plasmodium* species among different birds in New Zealand, Colombia, Bulgaria, and the United States differs between 20% and 32% (8-11) while based on the other reports prevalence rate of the parasites in Japan, Costa Rica, and Alaska was less than 10% (12-14). In a Cross-sectional study conducted by Zhang and colleagues (15), 7.8% of captured birds were infected with some species of *Plasmodium*. A study performed in Brazil showed that the level of infection with species of *Plasmodium* was 16.5% (16), where as this rate in the present study was 1.5% in considered birds for both of provinces. Moreover, this range can differ even in one country. It is important to note that the parasite prevalence and diversity will change with some geographical features such as temperature, climate, humidity, the prevalence of the vector and so on. In Iran, various research showed different results related to study areas. Yousefi and colleagues (17-18) could isolate *Haemoproteus* spp from a pigeon in the north of Iran. Dehghani Samani et al. (19) found that *Haemoproteus* infection among the old pigeons was higher than the younger pigeons in the west of Iran. Shemshadi and colleagues (20) reported that the rate of parasitic infection was 6.1% among the Caspian coastal birds in the north of Iran. Moreover, there are another information about existence of avian malaria in Iran. In this published data authors declared that the rate of infected birds was as follow: turkeys (10.2%), hens/roosters (1.7%), pigeons (6.1%) and migratory waterfowl (6.4%). These published data were recorded from Fars, Isfahan, and Mazandaran Provinces (21).

In the present study, microscopic examination revealed *Haemoproteus* spp infections in 25 samples (7.35%) of the 340 samples, also

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7 samples (14%) of the 50 suspicious and negative samples were positive as *Haemoproteus* and *Plasmodium* using PCR test. Regarding these two methods, the microscopic method is easy and cost-effective and thus it is the most common method for diagnosing *Plasmodium* and *Haemoproteus* species. However, it may sometimes show false negative results due to the low level of parasites in birds' blood, inappropriate staining, or presence of artifacts. In this concern, molecular analyses might be complementary methods for the microscopic of the diagnostic method. So, further studies are required for adjusting and preparing stable conditions of those analyses. The present study revealed the existence of malaria parasites and other haemosporidia in birds in Iran and it is a necessity to provide further studies to establish new prevention strategies to block transmission of the parasites in birds' community. PCR examination showed that there are more infections in the birds when researchers employ molecular methods. As it was implied before, assessment was based on size of the bands in PCR examination but considering that gene sequencing is a reliable way to confirm the presence of parasites, it is suggested to design a new study in this field.

Conclusion

Study on avian malaria in addition to knowledge of the condition of the disease among birds, can provide appropriate strategies for preventing further spread of the disease. Results of this study prompts scientist to conduct more studies in the field of avian malaria particularly in the studied areas.

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Ethical considerations

The study was approved by the joint Ethical Committees of Tehran University of Medical Sciences ethic no. IR.TUMS.SPH.REC. 1397.247.

Conflict of interest statement

Authors declare that there is no conflict of interest.

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