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Avian malaria associated with *Plasmodium* spp. infection in a penguin in Jeju Island

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Abstract : *Plasmodium* spp. in domestic and wild birds are microscopic, intracellular parasitic protozoa within the blood cells and tissues cause avian malaria. A 17-month-old Magellan penguin (*Spheniscus magellanicus*) with a clinical signs of anorexia, depression, and respiratory distress for 3 days was submitted to the Pathology Department of Veterinary Medicine, Cheju National University in October 2005. It was born and reared in the Jeju Island. Grossly, the liver was enlarged, pale and friable. The spleen was also enlarged with dark red coloration and friable. Histopathologically, the lesions in the liver were characterized by multifocal infiltration of macrophages and lymphocytes especially in perivascular regions. The schizonts of *Plasmodium* spp. contained up to 30 merozoites were found in numerous infiltrated mononuclear cells. Similarly, histiocytic cells were proliferated in red pulp of spleen and the schizonts were found in these cells. Numerous dark brown pigments were widely distributed in the liver and spleen. The result of the nested polymerase chain reaction clarified the causative agent of this case was *Plasmodium* spp.. This is the first report for the outbreak of avian malaria caused by *Plasmodium* spp. in a penguin that was born and reared in Jeju Island in Korea.

Keywords : avian malaria, nested PCR, penguin, *Plasmodium* spp., schizonts

Introduction

Plasmodium, *Haemoproteus* and *Leucocytozoon* spp. in domestic and wild birds are microscopic, intracellular parasitic protozoa within the blood cells and tissues and cause avian malaria throughout the world [6]. Their pathogenicity is variable but infections in highly susceptible species and age classes may result in death. *Plasmodium* spp. are transmitted from infected to uninfected birds by mosquitoes that serve as vectors. When infected mosquitoes bite a new host, infective stages of the parasites, sporozoites found in the salivary glands of these vectors gain entry to the tissues and blood of birds [2, 6, 17]. Immediately after they infect a bird, sporozoites invade the tissues and perform asexual reproduction to produce schizonts. And then a number of merozoites are released because of the breakdown of host cells. Merozoites invade other red blood cells and mature into infectious gametocytes [6].

Differences in the prevalence, geographic distribution, and host range of avian malaria infected with *Plasmodium* spp. are associated with habitat preferences of the bird hosts, the abundance and feeding habits within those habitats of suitable insect vectors, the specificity of the parasites for the avian host and innate physiological differences that make some avian hosts more susceptible than others [6, 11]. Penguins have little innate resistance and thus are highly susceptible to parasitism with *Plasmodium* spp. [9, 17]. *Plasmodium* spp. that highly affects penguins are transmitted by *Culex* mosquitoes [6].

Recently human malaria reemerged, and made a dramatic resurgence since 1993 in the demilitarized zone of Korea, and expanded towards eastern and southern part of the country year after year [8]. Just one case of avian malaria in the Humboldt penguins imported from Japan to the Farm-land Zoo was previously reported in Korea [3]. Here we described a case of *Plasmodium* spp. infection in a Magellan penguin born and reared at a theme park in Jeju Island.

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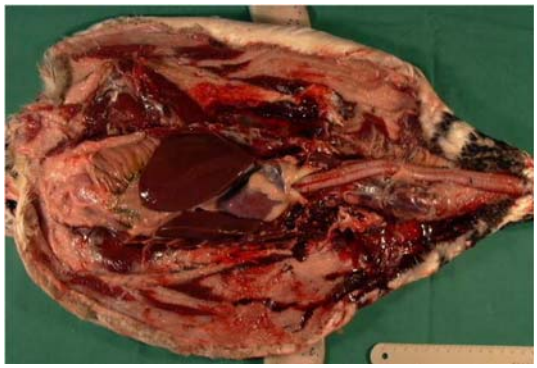

Fig. 1. Note enlarged liver of penguin.

Case Report

A theme park at the Jungmun Leisure Complex in Seogwipo-city, southern part of Jeju Province raised six Magellan penguins, 5 adults and 1 juvenile, for the indoor penguin exhibit. A 17-month-old Magellan penguin (*Spheniscus magellanicus*) that was born and reared at the theme park showed anorexia, depression, and respiratory distress for 3 days duration and then died in 28 October 2005. However all the other adult penguins did not show any clinical signs. The dead penguin was submitted to Pathology Department of Veterinary Medicine, Cheju National University for necropsy.

Grossly, major lesions were observed in liver and spleen. The liver was enlarged with round edge, pale discoloration and friable (Fig. 1). The spleen was also enlarged with dark red color and friable. The cut surface of spleen was granular. The lung was enlarged with purple inclining to red coloration and doughy consistency.

Tissue samples from the lungs, heart, liver, spleen, kidney, stomach, intestine and brain were fixed in 10% neutral buffered formalin for histopathologic examination. According to the standard tissue processing method, tissue samples were embedded in paraffin wax, cut into 2~3 μm-thick sections and stained with hematoxylin and eosin (H&E) for light microscopic examination.

Histopathologically, the lesions in the liver were characterized by multifocal infiltration of macrophages and lymphocytes especially in perivascular regions (Fig. 2). Most hepatic sinusoids were dilated and contained activated Kupffer cells. Many infiltrated macrophages and Kupffer cells had vacuolated cytoplasms and dark brown pigments. The schizonts of *Plasmodium* spp. that contained a number of merozoites were found in numerous infiltrated mononuclear cells and Kupffer cells (Fig. 2, Insert). Some hepatocytes also infected with parasitic schizonts. Similarly, histiocytic cells were diffusely infiltrated in red pulp of spleen (Fig. 3). White pulp of spleen showed moderated atrophy and mild lympholysis. Mature schizonts containing dot-like merozoites were frequently found in infiltrated histiocytic cells. Numerous dark brown pigments were widely distributed in whole spleen especially in histiocytes and macrophages. Capillaries

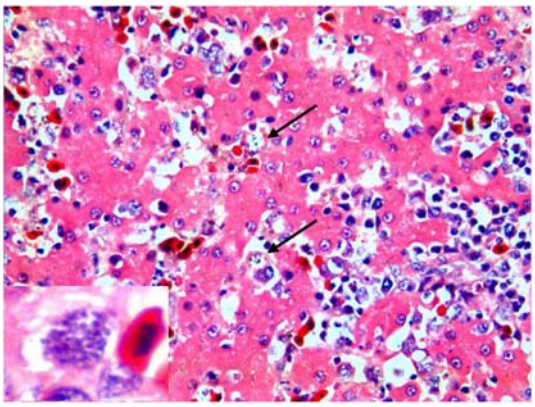

Fig. 2. Liver. Mononuclear cells infiltration in liver. Note schizonts in macrophages (arrow heads) and dark brown pigments (hemozoin, black arrows). H&E stain, ×400. Insert: Note schizont with many merozoites. H&E stain, ×1,000.

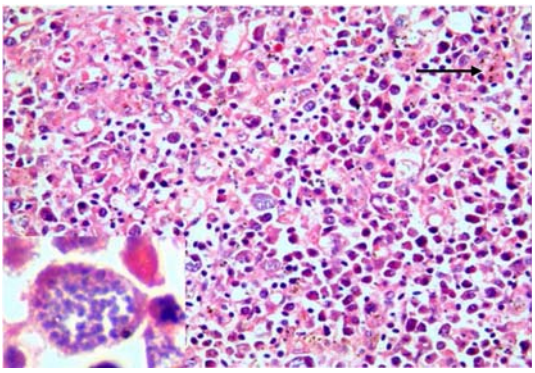

Fig. 3. Spleen. Diffuse infiltration of mononuclear cells in spleen.
Note schizonts in macrophages (arrow head) and dark brown pigments (hemozoin, black arrow). H&E stain, ×400. Insert: Note schizont with many merozoites. H&E stain, ×1,000.

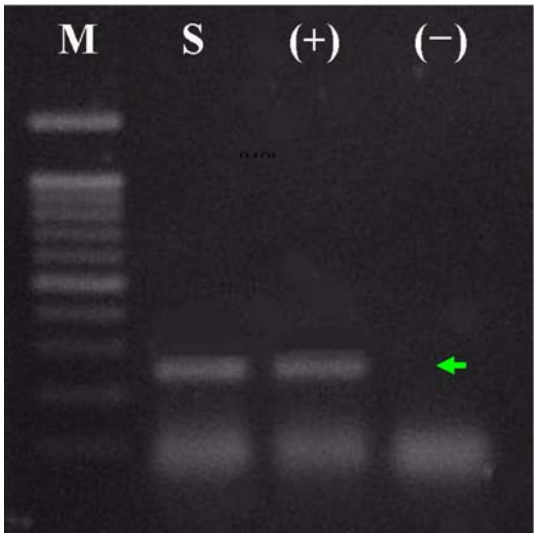

Fig. 4. Nested PCR products of *Plasmodium* spp. from tissue homogenates.
Lane M : 100 bp DNA ladder; lane S : 240 bp penguin tissue samples; lane (+): positive control; lane (−): negative control.

of lung were congested and eosinophilic material and some red blood cells were accumulated in bronchi and parabronchi. Reticular cells were proliferated diffusely in parenchyma of lung, particularly parabronchial areas.

Small tissue samples from the liver, spleen, lung, heart and kidney were homogenized with 10 ml of DNase RNase free distilled water (Invitrogen, USA). After centrifugation, 200 µl of supernatant were used for nested polymerase chain reaction (nested PCR) to detect *Plasmodium* spp. according to the method of Singh *et al.* [16]. Primers were designed based on the *Plasmodium* small subunit ribosomal RNA (ssrRNA) genes. First PCR produced an expected fragment of 1,600 base pairs (bp) with the forward primer rPLU1 (5'-TCAAAGATTAAGCCATGCAAGTGA-3') and the reverse primer rPLU5 (5'-CCTGTTGTTGCCTTAAAC TCC-3'). Nested PCR was performed with the forward primer rPLU3 (5'-TTTTTATAAGGATAACTACGGAA AAGCTGT-3') and the reverse primer rPLU4 (5'-TACCCGTCATAGCCATGTTAGGCCAATACC-3'). All PCR analyses were performed on the Thermal Cycler Dice TP600 (TaKaRa, Japan). Amplified products were visualized by staining with 0.5 µl/ml ethidium bromide on a 1.2% agarose gel. The result of electrophoresis clarified the positive band, 240 bp for *Plasmodium* spp. (Fig. 4).

Discussion

On the basis of the gross findings *Plasmodium* infection, *Haemoproteus* infection, and leucocytozoonosis were considered in the differential diagnosis. Definitive diagnosis of hemosporidian infections is dependent on microscopic examination of a stained blood smear or on an organ impression smear to detect the parasitized red blood cells [2, 6]. Because the penguin was died, we could not performed blood smear test in this case. Gross findings associated with *Plasmodium* spp. generally include enlargement of the liver and spleen, pericardial fluid, edema of the lung and the appearance of thin and watery blood [3, 6]. Parasites within the red blood cells produce an insoluble black pigment called hemozoin when they digest the host's hemoglobin. The hemozoin is deposited extensively in the host's spleen and liver tissue in this case. Hemozoin pigment is not produced in *Leucocytozoon* infection, therefore internal organs will not be as discolored and dark in necropsy [6]. However further differential diagnosis of *Plasmodium* from *Haemoproteus* may be difficult. Because *Haemoproteus* does not have an asexual stage in the blood, therefore diagnosis of a *Plasmodium* spp. infection is dependent on detection the presence of asexually reproducing stages of its life cycle, schizont, in the red blood cells or other tissue cells [6]. We successfully found the mature exoerythrocytic schizonts in liver and spleen contained up to 30 merozoites. These findings and characteristic histopathologic lesions in this penguin were resembled those previous reports [3, 5, 6]. Recently to overcome some limitations microscopic examination, PCR-base molecular assays have been developed for the detection and differentiation of malaria parasites [15, 16]. The nested PCR method used in this study have proved to be more specific and sensitive than conventional microscopy. Based on the gross findings, histopathological features and molecular method, the penguin was diagnosed as avian malaria by *Plasmodium* spp.

Avian malaria by *Plasmodium* spp. was highly infective in some species of birds and characterized clinically by acute course and high mortality [3]. Young birds are more susceptible than adults, and the most serious mortality generally occurs within the first few weeks of hatching [6]. It is well known that *Plasmodium* spp. are capable of causing severe anemia,

weight loss, and death in susceptible birds [1]. Survivors develop persistent, low-level infections in the blood and tissues that stimulate immunity to re-infection, and theirs immune system appears to be capable of reducing the number of parasites to sub-clinical levels [4, 6]. These survivors do not exhibit any signs of disease but they still have parasites. Thus they serve as reservoirs of infection, allowing the parasites to survive droughts and cold winter weather when vector populations have died off [6]. The recurrent recrudescences and relapses of malarial parasites in birds has been reported by many researchers but the causes is not clear [2, 14].

The mortality of penguins infected with *Plasmodoum* spp. is generally high [3, 5, 6], and it has been reported that stress and concurrent diseases including aspergillosis, bacterial enteritis and helminthiasis certainly contributed to the severe mortality eventually totaled 83% in penguins infected with *Plasmodium* (*P.*) *relictum* [5]. Since malaria in penguins was first reported in 1926 in a King penguin at the London Zoo, avian malaria had been accepted to be the most important parasitic agent causing severe epizootics at the Zoos in Europe and United States [7, 17]. In Korea, avian malaria in imported penguins from Japan was first described in 1984 [3].

Avian malaria in penguins is caused by *P. elongatum* and *P. relictum* [3, 6]. These parasites are transmitted by *Culex* mosquitoes. *Culex* mosquitoes have been distributed over most parts of the world including East Asia such as China, Japan, and Korea. Pigeons, canaries and sparrows can also be infected with *P. elongatum* and *P. relictum*, therefore these wild birds with an inapparent infection may serve as reservoirs of infection to penguins [6, 9]. The precise origin of the avian malaria remains unknown in this case. Recently, 42% (76/181) wild birds were positive for *Plasmodium* spp. in Korea [10]. Because Jeju Island is the warmer parts of the Korea, infected vectors may be well preserved in winter season. Mountain Halla National Park in central area and three birds wintering areas in western or eastern areas are located in Jeju Island. According to survey, 72 species and 133 species of birds were lived in Mt. Halla and wintering areas, respectively [12, 13]. Because of unique climate condition and high wild bird population, potential risk of epizootic avian malaria may be increase in Jeju Island. So epidemiological survey on avian malaria by infection of *Plasmodium* spp. should be performed in Korea including Jeju. In addition, breeding grounds of penguins should prevent from contacting with wild birds and mosquitoes. To reduce avian malaria in wild birds, highly control measures for mosquitoes should be warranted in Jeju Island.

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