AVIAN MALARIA IN PENGUINS.
TWO CASE REPORTS IN AFRICAN PENGUINS (*SPHENISCUS DEMERSUS*)

Marta ATTINI[1], Manuela ZANETTI[1], Carola CURCIO[1], Giada TRAMONTANO[1], Arianna TORRI[1], Francesco GRANDE[2], Raffaella CAPITELLI[3], Paola ROCCABIANCA[4], Gabriele GHISLENI[1]

1 BiEsseA Laboratorio analisi veterinarie, an Antech company, Milan, Italy; 2 Loro Parque Fundación - Avenida Loro Parque sn - 38400 Puerto de la Cruz, Tenerife, Spain;
3 CSV-Labvet, Cornaredo, Italy; 4 Department of Veterinary Medicine (DIMEVET), University of Milan, Milan, Italy

Background

Avian Malaria (AM) is a mosquito-borne disease of birds caused by haemoprotozoa belonging to the genus *Plasmodium* [2,3,4]. Over 50 species of *Plasmodium* parasites have been recognized, differing for host range, geographic distribution, competent vectors, reservoirs, and pathogenicity [1,6,9]. AM life cycle begins when a mosquito (Culicidae) injects *Plasmodium* sporozoites into the specific host, inducing exoerythrocytic merogony in reticuloendothelial cells [2,6,9,10]. Meronts undergo asexual multiplication to form numerous smaller mononuclear structures, the merozoites (Mz). Once the host cell ruptures, Mz are released into the bloodstream, invade the red blood cells and develop into trophozoites, which then grow to become erythrocytic meronts (erythrocytic cycle) [2,9]. Some of these Mz enter new red blood cells and transform into sexual gametocytes [9]. Generally, AM is subclinical in native birds that have co-evolved with the protozoa; contrarily, it causes significant mortality in captive birds, predominantly in warmer months [2,4]. Penguins, especially the family Spheniscidae, are highly susceptible to infection. In naïve individuals, *Plasmodium* species mainly elicit acute disease with minimal nonspecific to absent clinical signs such as appetite and weight loss and lethargy. Moreover, because of the low parasitemia, hemolysis is not severe enough to induce clinical overt anemia [2,3].

Objective

To outline the clinical signs and pathology of AM in two young females of African penguin (*Spheniscus demersus*) living in an openair colony.

Material and Methods

Two penguins died within a few days of the onset of non-specific clinical signs. CBC, biochemistry, and electrophoresis were performed before death. Necropsy was performed, and main organs were fixed in 10% buffered formalin and routinely processed for histology. On selected organs, microbiological cultures and ELISA for exotoxins of *Clostridium Perfrigens* were carried out, as well as conventional PCR for *Plasmodium* spp. targeting the mitochondrial cytochrome-B gene (Cyt-B). Cytological samples, obtained by tissue's imprint, were stained with May-Grünwald and Giemsa (MGG).

Results

Biochemical and electrophoretic results were unremarkable in both penguins. CBC values are reported in Table 1. On blood smear evaluation, neither parasitemia nor significant abnormalities were detected, except for a marked dyserythropoiesis and a severe heteropenia (case 1), and moderate toxic changes in heterophils (case 2). Gross necropsy reveals severe splenomegaly and hepatomegaly, mild nephromegaly, pulmonary oedema, pneumonia, multifocal epicardial petechial haemorrhages (case 1 and 2), hydropericardium (case 1), in animals in good body condition.
Histologically, interstitial pneumonia, severe periportal lymphoplasmacytic hepatitis and splenic lymphoreticular hyperplasia with intralesional protozoa were found in both cases. Cytologically, the pericardial fluid was acellular and consisted of a protein-rich transudate. No parasites were detected.
Cytology of the liver, spleen, and bone marrow revealed many meronts, free (Figures 1 and 2) and within the cytoplasm of macrophages (Figure 1, insert). These meronts were small roundish thin-walled bodies (20 µm in their largest diameter) containing small number of merozoites (less than 50). From all fresh tissue samples, microbial cultures and ELISA for exotoxins were negative. The suspicion of AM infection was confirmed by PCR on lung and brain tissues with amplifications of *Plasmodium* spp. mitochondrial DNA.

Table 1: Hematological results of two cases of AM with reference values [5,7]; abnormal values in bold red

PARAMETER	CASE 1	CASE 2	REFERENCE VALUES
RBC (x10⁶/µL)	1.73	1.67	(1.56 – 2.08)
Hgb (g/dL)	19.7	**14.9**	(16 - 20.8)
HCT (%)	51	**36**	(40 - 52)
MCV (fL)	**294**	215	(215 - 287)
MCH (pg)	113.8	89.2	(86.8 - 115.4)
MCHC (g/dL)	38.6	41.3	(36.5 – 43.5)
WBC (x10³/µL)	**6.7**	22	(9.3 – 26.1)
Heterophils (x10³/µL)	**1.34**	15.84	(6.71 – 17.16)
Lymphocytes (x10³/µL)	4.69	4.84	(3.58 – 15.07)
Monocytes (x10³/µL)	0.67	**0.88**	(0 – 0.82)
Eosinophils (x10³/µL)	0	**0.44**	(0 – 0.12)
Platelets (x10³/µL)	17.1	11.2	(5 – 19)

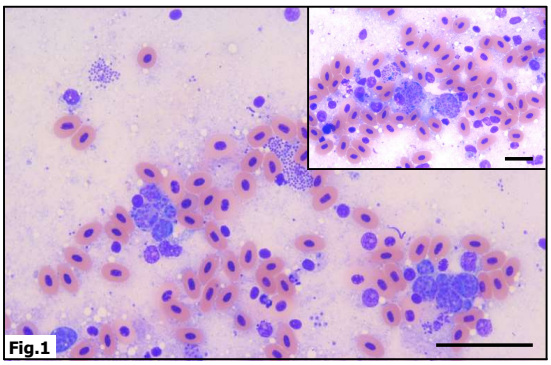
Fig.1

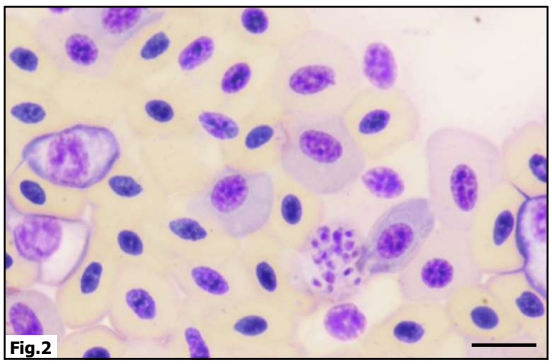
Fig.2

Figure legends
Fig. 1: Liver, impression smear case #1. MGG, Bar=50µm. Moderate cellularity, between hepatocytes, several exoerythrocytic free meronts filled with mature dispersed merozoites (<1µm of diameter) of *Plasmodium* spp.
Insert: Liver, impression smear case #1. MGG, Bar=20µm. Merozoites of *Plasmodium* spp. were found within the cytoplasm of two macrophages.
Fig. 2: Spleen, impression smear case #2. MGG, Bar=10µm. Between erythrocytes and polychromatophilic immature RBCs, one meront containing many stippled merozoites of *Plasmodium* spp. characterized by roundish to oval bodies and prominent basophilic nuclei.

Conclusions

As reported in the literature and due to the acute clinical course of the disease and low-level parasitemia in penguins, the diagnosis of AM is frequently obtained post-mortem by PCR. Gross pathology and histopathological examination of affected organs are useful diagnostic means to confirm malaria however, as in our cases, lesions are frequently nonspecific. Cytology is very helpful although not fully specific as exoerythrocytic meronts and "malaria pigment" (hemozoin granules) can be mistaken with other protozoan cysts or post-mortem artifacts on light microscopy [2,3]. For such reasons, PCR is considered the most sensitive diagnostic method, and primers targeting the mitochondrial cytochrome B gene (Cyt-B) are the most reliable [3,8]. Serologic ELISA tests can be applied to evaluate exposure, but they are basically of little value for the definitive diagnosis because penguins do not have time to mount the specific humoral immune response as they die during the acute phase of the disease [3]. Enabling better visualization of meronts of *Plasmodium* in birds affected with AM, cytology can represent a rapid and useful tool for *Plasmodium* spp. detection via direct impression smears from cut organ surfaces at post-mortem examination, but it may also assist in vivo diagnosis through endoscopic ultrasound-guided fine needle aspiration (EUS-FNA) of affected organs.

References

[1] Espinaze MPA, Hui C, Waller L, Dreyer F, Matthee S. Parasite diversity associated with African penguins (*Spheniscus demersus*) and the effect of host and environmental factors. Parasitology, 146(6): 791-804, 2019
[2] Grilo ML, Vanstreels RET, Wallace R, García-Párraga D, Braga ÉM, Chitty J, Catão-Dias JL, Madeira de Carvalho LM. Malaria in penguins - current perceptions. Avian Pathol, 45(4): 393-407, 2016
[3] Ings K, and Denk D. Avian Malaria in Penguins: Diagnostics and Future Direction in the Context of Climate Change. Animals (Basel), 12(5): 600, 2022
[4] Iurescia M, Romiti F, Cocumelli C, Diaconu EL, Stravino F, Onorati R, Alba P, Friedrich KG, Maggi F, Magliano A, Ermenegildi A, Carfora V, Caprioli A, De Liberato C, Battisti A. *Plasmodium matutinum* Transmitted by *Culex pipiens* as a Cause of Avian Malaria in Captive African Penguins (*Spheniscus demersus*) in Italy. Front Vet Sci, 8: 621974, 2021
[5] Mazzaro LM, Meegan J, Sarran D, Romano TA, Bonato V, Deng S, Dunn JL. Molt-associated changes in hematologic and plasma biochemical values and stress hormone levels in African penguins (*Spheniscus demersus*). J Avian Med Surg, 27(4):285-293, 2013
[6] Paez AM, Chalkowski K, Zohdy S, Willoughby JR. Management of avian malaria in populations of high conservation concern. Parasit Vectors, 15(1): 208, 2022
[7] Parsons NJ, Gous TA, Schaefer AM, Vanstreels RET. Health evaluation of African penguins (*Spheniscus demersus*) in southern Africa. Onderstepoort J Vet Res, 83(1): e1-e13, 2016
[8] Richard FA, Sehgal RNM, Jones HI, Smith TB. A comparative analysis of PCR-based detection methods for avian malaria. J Parasitol, 88(4): 819-822, 2002
[9] Rivero A and Gandon S. Evolutionary Ecology of Avian Malaria: Past to Present. Trends Parasitol, 34(8): 712-726, 2018
[10] Valkiūnas G and Iezhova TA. Exo-erythrocytic development of avian malaria and related haemosporidian parasites. Malar J, 16(1): 101, 2017