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Histopathology of protozoal infection in animals: a retrospective study at the University of Philippines College of Veterinary Medicine (1972-2010)

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Summary

The authors describe the first parasitological survey of protozoal infections on tissue slide sections of field cases processed at the histopathology laboratory of the College of Veterinary Medicine (CVM) at the University of the Philippines Los Baños (UPLB). Over 80% of the field cases were from Region 4 (CALABARZON) and the rest were equally distributed from other areas of the Philippines, namely: Region 2 (Cagayan Valley), Metropolitan Manila (National Capital Region), Region III (Central Luzon) and Region VI (Western Visayas). Histopathological analyses of tissue sections from 51 archived cases (1972-2010) of parasitic aetiology were performed. Microscopic examination of a total of 286 histopathological slides revealed the presence of several protozoa, including sarcosporidiosis, hepatic coccidiosis, intestinal coccidiosis, balantidiosis and leucocyto-zoonosis. In addition, the finding of *Balantidium* and *Sarcocystis* may have zoonotic implications and can therefore be used as markers of public health importance.

Keywords

Histopathology, Parasite, Parasitism, Protozoa, Philippines, Public health, Tissue section.

Istopatologia da infezione protozoaria negli animali: uno studio retrospettivo (1972-2010) presso l'Università delle Filippine nel College di Medicina Veterinaria

Riassunto

Gli autori descrivono la prima indagine parassitologica delle infezioni protozoarie su vetrino attraverso sezioni di tessuto, di casi trattati in campo, presso il laboratorio di istopatologia del Collegio di Medicina Veterinaria (CVM) attivo nell' Università delle Filippine a Los Baños (UPLB). Oltre l'80% dei casi provenivano dalla Regione 4 (CALABARZON) e il resto dei casi proveniva equamente da altre zone delle Filippine, e precisamente: dalla Regione 2 (Cagayan Valle), Metropolitan Manila (National Capital Region), dalla Regione III (Central Luzon) e dalla Regione VI (Western Visayas). Sono state effettuate analisi istopatologiche di sezioni di tessuto provenienti da 51 casi archiviati (1972-2010) con eziologia parassitaria. L' esame microscopico su un totale di 286 vetrini istopatologici, ha mostrato la presenza di alcune malattie protozoarie, tra cui la Sarcocistosi, la Coccidiosi epatica, la Coccidiosi intestinale, la Balantidiasi e la Leucocitozoonosi. Inoltre, la presenza del protozoo Balantidium e Sarcocystis, può avere implicazioni zoonotiche ed essi possono quindi essere usati come importanti indicatori nella salute pubblica.

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Parole chiave
Istopatologia, Parassita, Parassitismo, Protozoi, Filippine, Salute pubblica, Sezioni di tessuto.

Introduction

Infections caused by protozoan parasites are widely known to infect various hosts in the Philippines and they are responsible for many diseases observed in both domestic and wild animals, as well as in birds and humans. Certain species of medical importance have been investigated with considerable care in the country, especially the malarial parasites, the dysentery amoeba, *Entamoeba histolytica* and the dysentery ciliate, *Balantidium coli* (17). According to a previous study that was conducted approximately over eight decades ago, several protozoan parasites were found from different animals in the Philippine Islands. This includes *Eimeria* in rabbits, bats and python, *Isospora* in the Ruddy kingfisher (*Halcyon coromanda*) and Crested Myna (*Acridotheres cristatellus*) and Pied Triller or Lalage (*Lalage nigra*) and *Haemoproteus* in the Dollarbird (formerly Broad-billed Roller) (*Eurystomus orientalis*), Besra (formerly Philippine sparrowhawk) (*Accipiter virgatus*), Philippine oriole (*Oriolus steerii*), Philippine coucal (formerly Java coucal) (*Centropus viridis*) and Spotted Wood-kingfisher (formerly Lindsay's kingfisher) (*Actenoides lindsayi*). In monkeys, several *Endamoeba* and *Trichomonas* species, *Endolimax*, *Dientamoeba*, *Chilomastix*, *Giardia lamblia* and *B. coli* were also listed (17).

Generally, there are no specific clinical signs associated with the infection in animals (10, 43), especially protozoan infection in nature. Frequently manifested signs include weight loss, inappetence, weakness and gastro-intestinal signs, such as diarrhoea. These are often seen in immunocompromised animals, or young animals with heavy infestations (18, 43); however, healthy animals may show various signs as well.

Several methods are used to diagnose the presence of protozoa. Basically, microscopic examination of blood and faecal samples are the most common methods of examination performed to detect protozoa at the College of Veterinary Medicine (CVM) of the University of the Philippines Los Baños (UPLB). Another useful method but one which is seldom applied or only performed on a case-to-case basis at the CVM-UPLB is histopathology of tissue sections. Upon microscopic examination of tissues, protozoa may be seen in the intestinal crypts, or free in the lumen of the gastrointestinal tract and in organs of extra-intestinal origin, such as the liver, lungs, muscle and bile duct, among others (2, 17, 18, 21, 23, 40). Some examples of protozoa found in the blood are *Plasmodium* spp. (5, 49), *Leukocytozoon* spp. (50), *Trypanosoma* spp. (6, 7, 8, 13), those found in faeces are *Giardia* (22) and *Balantidium* (15); *Cryptosporidium* spp. (24) and species found in extra-intestinal origins are *Eimeria grallinida* and *E. stiedae* in the liver and bile ducts (41); *Isospora serine* is found in the respiratory system (12) and *Sarcocystis* spp. which are usually found in the muscles, brain and in other predilection sites, depending on the species (14, 38).

The histopathological examination of tissues can be of great value as a diagnostic tool because the finding of the protozoan organism is definitive. Since suspected protozoal cases are not often diagnosed by histopathological examination at the UPLB-CVM, this study therefore dealt with a retrospective review of protozoan infections through histopathological examinations.

The paper reports on the different species of protozoan parasites found upon histo-pathological examination. Results of the examination revealed an overall percentage of 9.8% positive for protozoa. In addition, the finding of *Balantidium* (2%) and *Sarcocystis* (2%) suggests zoonotic implications and may therefore be used as markers of public health importance.

Materials and methods

Data collection

Handwritten records stored in the logbooks of the Histopathology Laboratory of the Department of Veterinary Paraclinical Sciences (DVPS) at the CVM-UPLB were searched for keywords 'parasitism, protozoa or specific

names of parasitic infections' from 1972 to 2010. The histopathology reports of this cohort of cases were included as samples for the study. Respective control numbers of the cases were correlated with the slides and these sections were set aside for microscopic examination.

Histopathological examination

The different tissue samples were processed using the routine paraffin technique for histological examination. Briefly, tissue samples were fixed in 10% formalin solution to arrest tissue decomposition or degeneration. Thereafter, tissues were dehydrated with increasing concentrations of alcohol. A portion of each sample was individually embedded in paraffin and serially sectioned (3 µ -10 µ) using American Optical 820 Rotary Microtome® (Scientific Instruments Division, Buffalo, New York), followed by slide mounting and haematoxylin and eosin tissue staining. Processed samples were individually examined under low power and high power magnification and oil immersion objective when necessary. A systematic approach was employed in the examination of the entire slide. Photomicrographs were obtained using a Nikon Eclipse E-200 microscope with DS-Fi1-L2 colour camera head and LCD monitor (Nikon, Tokyo).

Results

A majority of field cases (83.3%) were from Region 4 (CALABARZON) and the rest were equally distributed at 4.2% in other areas of the Philippines, namely: Region 2 (Cagayan Valley), Metropolitan Manila (National Capital Region), Region III (Central Luzon) and Region VI (Western Visayas).

The histopathological examination of 286 tissue sections disclosed a variety of protozoal infections. Amongst the cases obtained, hepatic coccidiosis (Fig. 1), intestinal coccidiosis (Fig. 2), sarcosporidiosis (Fig. 3) and balantidiosis (Fig. 4) can be transmitted orally and one, leukocytozoonosis (Fig. 5), requires an arthropod vector host.

Figure 1 shows several stages of the parasite, specifically macrogametocyte and microgametocyte, in the liver parenchyma of a rabbit. In addition, microscopic lesions, such as severe biliary hyperplasia, were observed. Similarly, in Figure 2, partly destroyed intestinal wall with different stages of coccidian parasite were observed. Occupying the intestinal lining were several macrogametocyte, microgametocyte, immature oocysts with zygote and schizonts with many elongated merozoites. In the case of Figure 3, sarcocysts or muscle cysts were displayed. Subsequently, crescent-shaped slender bradyzoites were revealed at higher

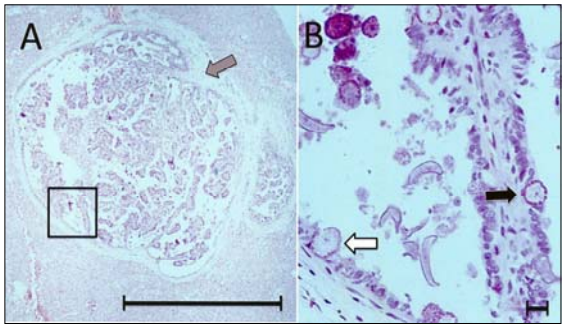

Figure 1
Liver of rabbit with *Eimeria stiedae* infection
(A) Grey arrow shows convoluted hyperplastic bile duct
Area enclosed a box (bar = 1 mm)
(B) Black and white arrows indicate the microgametocyte and microgametocyte, respectively (bar = 1 µm)
The tissue sections were processed using H & E stain

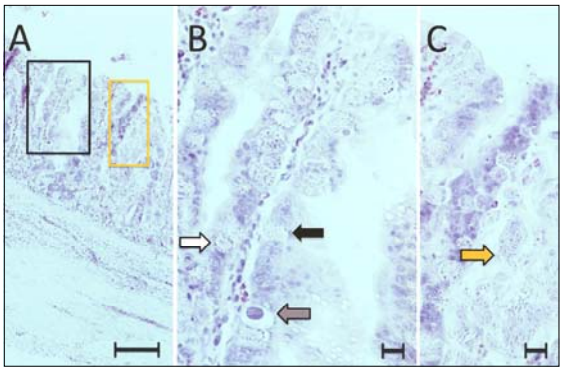

Figure 2
Intestinal coccidiosis
(A) Black box includes the region of (B) and yellow box is the designated zone for (C) (bar = 10 µm)
(B) Black, white and grey arrows indicate the microgametocyte, macrogametocyte and immature oocyst with zygote, respectively (bar = 1 µm)
(C) Yellow bar represents a schizont with several merozoites (bar = 1 µm)
The tissue sections were processed using H & E stain

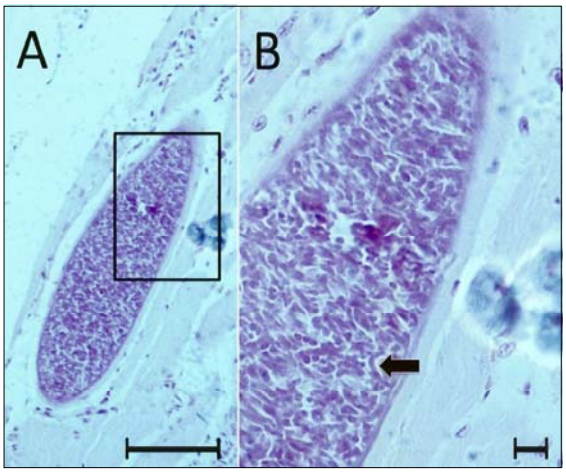

Figure 3
Sarcocystis infection in the muscle
(A) Sarcosyst (bar = 10 µm)
Black box represents the area of (B)
(B) Black arrow indicates the banana-shaped bradyzoites (bar=1 µm)
The tissue section was processed using H & E stain

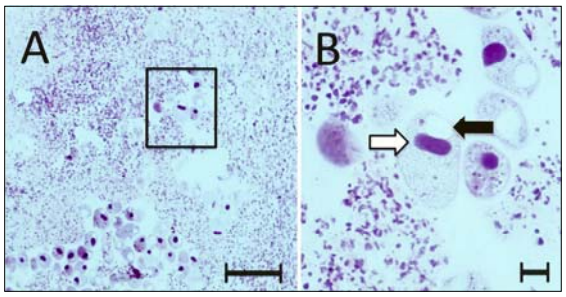

Figure 4
Balantidium infection in the caecum
(A) Several trophozoite forms in the tissue section (bar =10 µm)
Black box denotes the area of (B)
(B) Black and white arrows indicate the contractile vacuole and macronucleus of the trophozoite form (bar =1 µm)
The tissue section was processed using H & E stain

magnification. Figure 4, on the other hand, illustrated parasite stages in the gut of swine. A morphological description of the ovoid-shaped parasite stage with sausage-like nucleus and contractile vacuole (trophozoite form) was recorded. Lastly, Figure 5 revealed two large schizonts or megaloschizonts which were encapsulated in connective tissues, indicating one of the typical lesions of leukocytozoonosis.

Discussion

In Figure 1, several ova of coccidian parasite were found in the liver parenchyma of a rabbit. The liver section examined showed distinct microscopic lesions, namely: severe biliary hyperplasia with numerous intra-epithelial coccidia which is highly indicative of hepatic coccidiosis.

E. stiedae, the causative agent of hepatic coccidiosis, is a common disease of rabbits that can result in severe hepatic injury and death in juveniles and neonates (Fig. 1). Gross examination of the liver will therefore show multifocal to coalescing, linear, yellow to grey nodules which are considered characteristic features of classical appearance of hepatic coccidiosis (1, 41). On the other hand, the microscopic lesion associated with hepatic coccidiosis is unique and nearly pathognomonic because *E. stiedae* causes proliferation of bile duct epithelial cells. Thus, affected livers contain multifocal, well-demarcated, linear, occasionally branching, bosselated, yellow to pearl-grey lesions that reflect the course of the biliary tree (2, 19). In addition, the feature of the oocyst stages in ovoid or ellipsoidal shapes, flattened at one end and approximately 35 µm-40 µm × 23-28 µm is characteristic of the parasite infection (17). Consequently, the description of the ova stage of the parasite obtained matched that of the *E. stiedae* morphological picture.

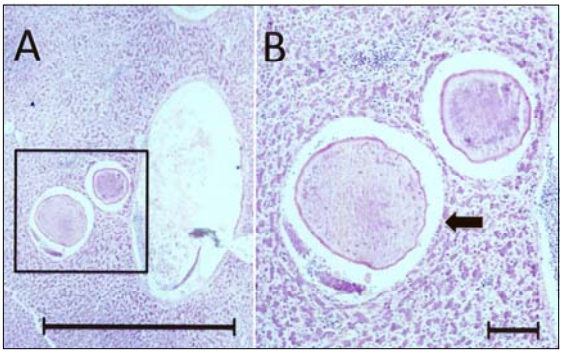

Figure 5
Hepatic tissue of chicken with *Leucocytozoon* infection
(A) Black box denotes the area of (B) (bar =1 mm)
(B) Black arrow represents megaloschizont stage (bar = 10 µm)
The tissue section was processed using H & E stain

The parasite, *E stiedae*, was first documented in the Philippines in the 1930s. Infected animals were rabbits raised by the University of

Philippines CVM Pandacan, Metropolitan Manila (17). In this respect, the present study is in agreement with previous reports and confirms *E. stiedae* infection in the liver section of a rabbit.

Apart from hepatic coccidian species, intestinal coccidiosis was also observed (Fig. 2). Several coccidia usually penetrate the wall of the intestine and develop at the expense of the cells of the intestinal lining. The presence of oocyst in the faeces was indicative that the animal was infected (17). Figure 2 shows the denuded intestinal wall and different stages of coccidian parasite. The stages observed include the following: macrogametocyte, microgametocyte and immature oocysts with zygotes. In addition, several schizonts containing numerous elongated merozoites were documented.

Intestinal coccidiosis is caused by several species in the family Eimeriidae (43). In the Philippines, the most frequently encountered species in the past has been those belonging to *Eimeria* and *Isospora*. Oocysts of an *Eimeria* species have been reported in python, rabbits and Philippine bats. Oocysts of *Isospora* have also been documented among birds captured in the Philippines, namely: the Ruddy kingfisher, Pied Triller or Lalage and Crested Myna. Although cases of avian coccidiosis were detected in wild birds in the previous study, it was however advocated that wild avian species were not heavily infected compared to their domestic counterparts (17). In the study conducted by Tubangi (45), the oocysts of the following were encountered:

- *E. canis* and *Isospora rivolta* in dogs
- *Isospora* sp. in cats
- *E. tenella* in chickens
- *E. debliecki* in swine
- *E. zurnii*, *E. smithi* and *E. bukidnonensis* in cattle
- *E. zurnii* and *E. smithi* in Carabao (*Bubalus bubalis* L.)
- *E. faurei* in sheep and goats.

Similarly, *E. arloingi* and *E. granulosa* were also documented in goats (39).

Muscle cysts are microscopically illustrated in Figure 3. A cross-section of the cysts exhibited crescent-shaped slender bradyzoites. The third reported case was identified as *Sarcocystis* spp. infection or sarcosporidiosis.

The pathology of the disease has two facets, namely: a rare invasive form with vasculitis and myositis and an intestinal form that presents with nausea, abdominal pain and diarrhoea. The predominant histological lesions are lymphoplasmacytic and histiocytic inflammation and of protozoal merozoites and schizonts in the muscles (14, 38).

The disease sarcosporidiosis has been reported in the Philippines for many years.

According to Arambulo *et al.* (3), *Sarcocystis* (*S. blanchardi*) was first documented in the Philippines from a Carabao in Luzon Island by Boynton in 1916. He further cited that it was detected in slaughtered meat from Pasig City (1926) and afterwards found in a cafeteria at the University of the Philippines (1960). Consequently, the incidence of *Sarcocystis* infection from the Manila abattoir became evident (3) and tropozoite stages in meat (beef) sold around Metropolitan Manila were similarly reported (44). This was followed by the works of Manuel *et al.* (37) who reported that sarcocyst of different sizes were composed specifically of macrocysts and microcysts. In addition, it was concluded that there were three *Sarcocystis* species that occurred in Philippine Carabao, namely: *Sarcocystis levinei*, *S. cruzi* and *S. fusiformis*. Furthermore, a case of *Sarcocystis* infection was also described in mammals and reptiles. Specifically, Carlos and Schaffer (9) observed *Sarcocystis* infection in rats, whereas a recent incident of *S. singaporensis* in python from Luzon has been documented by Jäkel *et al.* (20). Consequently, there are also *Sarcocystis* species that infect humans.

Three species of *Sarcocystis* can be found in humans. The first of these, *S. lindemanni* utilises humans as an intermediate host. The other two species, *S. porcihominis* and *S. bovihominis* (*S. hominis*) requires the human being to be the definitive or final host (43). A previous study using light microscopy suggested that the different sarcocysts examined from cattle were *S. cruzi*, *S. bovifelis*

and *S. bovihominis* (11). Since it has been suggested that a zoonotic species is present in the Philippines, the possibility that the data presented, which was obtained almost 40 years ago, is a zoonotic species cannot be ignored.

Figure 4 shows the parasite stages in the cross-section of intestines from swine. As mentioned by Manlove (27), a large proportion of swine in and about Manila are parasitised with *Balantidium* species. Furthermore, domestic and wild pigs examined in a previous study were infected by *B. coli* (17).

Conversely, the morphological features of the parasites in the tissue samples examined in this study closely resembled previous reports wherein an ovoid-shaped ciliated parasite stage with sausage-like nucleus and contractile vacuole representing trophozoite was documented. *Balantidium* species has two developmental stages, a trophozoite stage and a cyst stage. In trophozoites, the two nuclei are visible. The macronucleus is long and sausage-shaped, and the spherical micronucleus is nested next to it, often hidden by the macronucleus. Specifically of zoonotic importance, *Balantidium* is the only ciliated protozoan known to infect humans (15, 17, 43, 48). The commensalistic parasite has a wide geographic distribution and is extensively found among mammals (25). Moreover, the parasite is generally believed to be found ubiquitously around the world and is present whenever there are pigs present in the area (42).

Subsequently, this parasite species was determined to be endemic in the Philippines. A previous study showed that 18.18% (8/44) of monkeys examined were found to be infected with *B. coli* or balantidiosis.

A pioneering study on *B. coli* in the Philippines as cited by Arean and Koppisch (4) was performed by Strong in 1904. From several cases, including seven of his original works, he published the earliest account of *B. coli* infection in the Philippines. Additional data was added by other research workers five years later (Bowman, 1909 as cited by Arean and Koppisch [4]). It was demonstrated that nearly 1% of the 4 000 people examined were positive for the infection. The report of Walker (46) also showed that there were 35 cases of infection in the Bilibid Prison and pointed out that the ciliate is common in the Philippines. In the work of Willets (47), 0.2% of patients examined in the University of the Philippines General Hospital (UP PGH) were found to have *Balanditium* infection. Three years later, Manlove (27) described two cases of balantidial colitis in patients at the UP PGH. Pathogenesis of the parasite is initiated upon invasion of the colonic mucosa and thereafter the parasite is distributed either locally or systemically (25). According to Strong (1904) and Bowman (1911) as cited by Manlove (27), one and two recorded deaths, respectively, were documented. In addition, Walker (46) showed that several positive individuals had been in contact with pigs.

The last case of protozoal infection in the study was observed in avian species. Figure 5 shows microscopic images of large schizonts or megaloschizonts containing merozoites which is one of the typical lesions of leukocyte-zoonosis. The classic pathology of infection with these parasites includes enlargement of the liver, spleen and pulmonary congestion and pericardial effusion. Grossly, megalo-schizonts appear as grey-white nodules found in the heart, liver, lung or spleen. Micro-scopically, there is ischemic necrosis and associated inflammation in the heart, brain, spleen and liver due to occlusion of blood vessels by megaloschizonts in endothelial cells. Ruptured schizonts may also induce granulomatous reactions in the surrounding tissues (16, 26, 29, 43). Lesions encapsulated with layers of connective tissue, which is characteristic of a granulomatous reaction, were also observed.

Over 40 years ago, leukocytozoonosis involving domestic birds in the Philippines was first reported by the Manuel (28). It started as a strange malady that inflicted very heavy losses on many poultry raisers in Central Luzon. Affected areas were Nueva Ecija, Pampanga, Tarlac, Pangasinan, Bulacan, Rizal, Cavite, Laguna and Batangas. It was later established that the average mortality rate was as high as 10% among young chickens (29).

Subsequently, an infectivity rate of 45.53% was also recorded (31). The arthropod vector hosts were biting midges (*Culicoides*) in which parasites multiply and eventually give rise to several sporozoites or infective stages (31, 43). The report on the occurrence of this parasite, paved the way for an evaluation of several drugs (sulfamonothoxine, pyrimethamine, clopidol, halofuginone and furazolidone) to determine their prophylactic value against leukocytozoonosis (31, 32, 33, 34, 36). Consequently, *Leucocytozoon* sp. (Andrewsi type) was also detected in the red blood cells of wild birds which include a Tricolored Munia (*Lonchura malacca)*, Scaly-breasted Munia (formerly Nutmeg Mannikins) (*Lonchura punctulata*) and three Luzon Rufous hornbills (*Buceros hydrocorax*) (30, 35).

Histopathological surveys of parasitic protozoa are of interest as they often reveal species that were previously unknown and thereby add to our knowledge of the distribution of the parasite. From 1972 to 2010, the percentage of parasitic cases was (51/1 460) or 3.5% whereas the confirmed protozoan cases were 0.34%. Since the result of positive protozoan infection was low, statistical analysis was deemed no longer necessary.

In instances where the disease examined is of low incidence, a retrospective study design is necessitated to initially address or identify such diseases. Otherwise, a prospective study design would necessitate extremely large cohorts and would be very expensive and time consuming. The disadvantages of a retrospective study are its inability to control exposure or outcome assessment and its strong reliance on others for accurate record-keeping. A major limitation of this study lies in the insufficient data (history) which accompanied each case. In particular, Figures 1 to 5 had no existing data and diagnosis relied solely on the histopathological examination.

Conclusions

Although in most cases it has been impossible for us to determine the specific identity of the protozoa found due to insufficient records, the histopathological examinations of tissues proved to be of great value as a diagnostic tool for protozoan diagnosis because the finding of the organism(s) is definitive. Furthermore, knowledge of this subject is of scientific, practical and public health importance. Although the results reported are fragmentary, they serve as the beginning of a large project and it is hoped that the study will stimulate further investigations.

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