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Diagnostic pathology of leucocytozoonosis in Banda Aceh, Indonesia

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Abstract

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Leucocytozoonosis is caused by *a Leucocytozoon* sp. infection, which is transmitted through the vector *Culicoides arakawae,* and results in lethargy, anemia, fever, paralysis, and even death in poultry. Numerous diagnostic methods, including clinical signs, blood smear examination, and polymerase chain reaction (PCR), have been developed; however, these methods tend to have certain limitations. This study, therefore, aims to observe gross pathology and histopathological lesions in broiler chicken organs with leucocytozoonosis within Banda Aceh, Indonesia, as a pathognomonic feature of the disease, to enable precise diagnosis. A total of 40 fresh broiler chicken cadavers were collected from several markets around Banda Aceh. The samples were then necropsied, and the appearance of all organs and tissues was observed. Subsequently, the organs and tissues with lesions were subjected to histopathological and parasitological examinations, then stained using hematoxylin-eosin. Gross anatomical examination results revealed hemorrhages in the pectoralis and femoralis muscles, trachea, liver, heart, lungs, proventriculus, intestine, thymus, bursa of Fabricius, spleen, kidney, and brain. The histopathological examination revealed the presence of schizonts and megaloschizonts in organs and tissues, accompanied by haemorrhage. In contrast, the parasitological method indicated that schizonts were present in the muscle and lungs. The study's findings are crucial in preventing the outbreak of the disease.

Keywords: gross anatomy; histopathology; leucocytozoon; parasite; schizont

Introduction

Leucocytozoonosis is a malaria-like disease caused by *Leucocytozoon* sp. and transmitted by black flies (*Simulium* sp), as well as biting midges (*Culicoides arakawae*) (Yu et al., 2001; Omori et al., 2008; Jumpato et al., 2019). Two leucocytozoon species commonly infect chickens: *Leucocytozoon caulleryi,* which is transmitted by *Culicoides arakawae*, *Culicoides circumscriptus,* and *Culicoides odibilis*, as well as *Leucocytozoon sabrazesi* (Pramual et al., 2021; Xuan et al., 2021). In Indonesia, *Leucocytozoon caulleryi* is transmitted to chickens by *Culicoides arakawa* (Suprihati et al., 2020). This disease is significant to the poultry industry because it reduces productivity and also has lethal effects on poultry livestock (Pramual et al., 2020).

Leucocytozoonosis is a vector-borne disease; therefore, its prevalence is significantly influenced by the occurrence of insects around the environment (Xuan et al., 2021). Tropical regions tend to have a higher prevalence of leucocytozoon, compared to the subtropics, because insects are present all

year round in the tropics, but are more active in spring within subtropical regions (Ozmen and Haligur, 2005). Pramual et al. (2020) reported seasonal abundance changes in adult black fly species, with the highest abundance observed in the dry season (March to May). This seasonality is probably related to the availability of suitable habitats for juvenile stages, considering adult females carry the infectious stage of the parasite (Suprihati et al., 2020).

The number of sporozoite-carrying vectors significantly influences the prevalence of the disease in that area (Win et al., 2020). Previous studies have reported the prevalence of Leucocytozoon within several areas in Indonesia. According to these reports, this prevalence is 14.2% in broiler chicken within Jombang, East Java (Arifiandani et al., 2019), 53.58% in native chicken within Bali (Here et al., 2017), and 30% in broiler chicken and 24% in duck within Aceh Province (Hanafiah et al., 2007).

The incidence of leucocytozoonosis is categorized into acute, subclinical, and chronic. Acute leucocytozoonosis occurs in various poultry groups; however, in young poultry, which is most sensitive to the disease, the effects are most severe, and the mortality rate can reach up to 80%. Subacute cases are also characterized by high mortality rates (Kaoud, 2017), as well as symptoms of decreased appetite, thirst, depression, dull hair, paleness, loss of balance, weakness, shortness of breathing, lethargy, paralysis, and anemia. The pathogenesis of this disease is rapid, and chickens either recover naturally or succumb to death (Swayne, 2013).

Generally, leucocytozoonosis cases in growing chickens tend to be subclinical. However, in layer chickens, the disease drastically reduces egg production and often takes about two months for production levels to return to normal (Kaoud, 2017). Chickens that survive leucocytozoonosis are bound to experience chronic infection, as well as impaired growth and production. The chronic form of infection typically exhibits no signs of bleeding. However, the chickens appear pale (anemic) for only a short time, excrete green diarrhea, experience a sharp reduction in growth and production, and sometimes lay eggs with soft shells and spots (Nath et al., 2014).

According to Zhao et al. (2016), the degree of disease severity varies with the incidence of disease on a farm. Clinical symptoms are influenced by the number of *Leucocytozoon* sp. that develop in the chicken's body, as well as the age and type of animal affected. Usually, chickens below one Month old that are infected with Leucocytozoon tend to die within 13 days of infection, while adult chickens tend to suffer from anemia and a greenish fecal color; however, some chickens do not show any symptoms.

The disease is often diagnosed based on clinical symptoms, including specific lesions, post-mortem abnormalities, and a history of the disease (Ramey, 2021). This diagnosis is also confirmed by examining blood smears with a microscope, Polymerase Chain Reaction (PCR), and serological tests, including the Enzyme-Linked Immunosorbent Assay (ELISA) (Kilpatrick et al., 2006; Win et al., 2020). Blood smear examination can identify blood protozoa in the gametocyte or merozoite phase using specific stains, such as Giemsa. Precise identification with this process alone tends to be difficult. Thus, a PCR examination is required to identify blood protozoa down to the species level (Jomkusing, 2021). However, PCR examinations are pretty expensive. This led to the use of necropsy, histopathology, and parasitology in diagnosing *Leucocytozoon* sp infection in chicken carcasses, through studying the pathognomonic symptoms of haemorrhage (petechiae), as well as schizonts and megaloschizonts in chicken organs and tissues (Nath et al., 2014). The necropsy, histopathology, and parasitology methods are significantly easier, less expensive, and more reliable; however, accurate diagnosis is required to develop these methods for effective treatment, transmission control, and disease management.

Material and Methods

Sample collection and gross anatomy examination

This study utilized 40 fresh broiler chicken cadavers randomly collected from various markets in Banda Aceh, Indonesia. All samples exhibiting clinical symptoms of hemorrhage, anemia, or green diarrhea were packaged and transported to the Pathology Laboratory of the Veterinary Medicine Faculty at Universitas Syiah Kuala, Indonesia. Subsequently, the samples were subjected to necropsy involving an anatomical pathology examination performed by meticulously observing the appearance of the organs and tissues. The lesions observed on the tissues and organs were then documented and collected for histopathological and parasitological examination.

Histopathological evaluation

For this evaluation, the tissues and organs were first rinsed with saline solution to remove any unwanted materials, then fixed in 10% Neutral Buffered Formalin (NBF) for at least 24 hours. Subsequently, the samples were soaked in 70% alcohol as a stopping point for 12 hours, then transferred to an alcohol series of 80%, 90%, 95%, and absolute for 2 hours each, to dehydrate the tissues. This was followed by clearing the tissues samples in xylene solution for 45 minutes and infiltrating the tissues with paraffin for 45 minutes, before embedding the tissues in the paraffin block. The tissues sam-

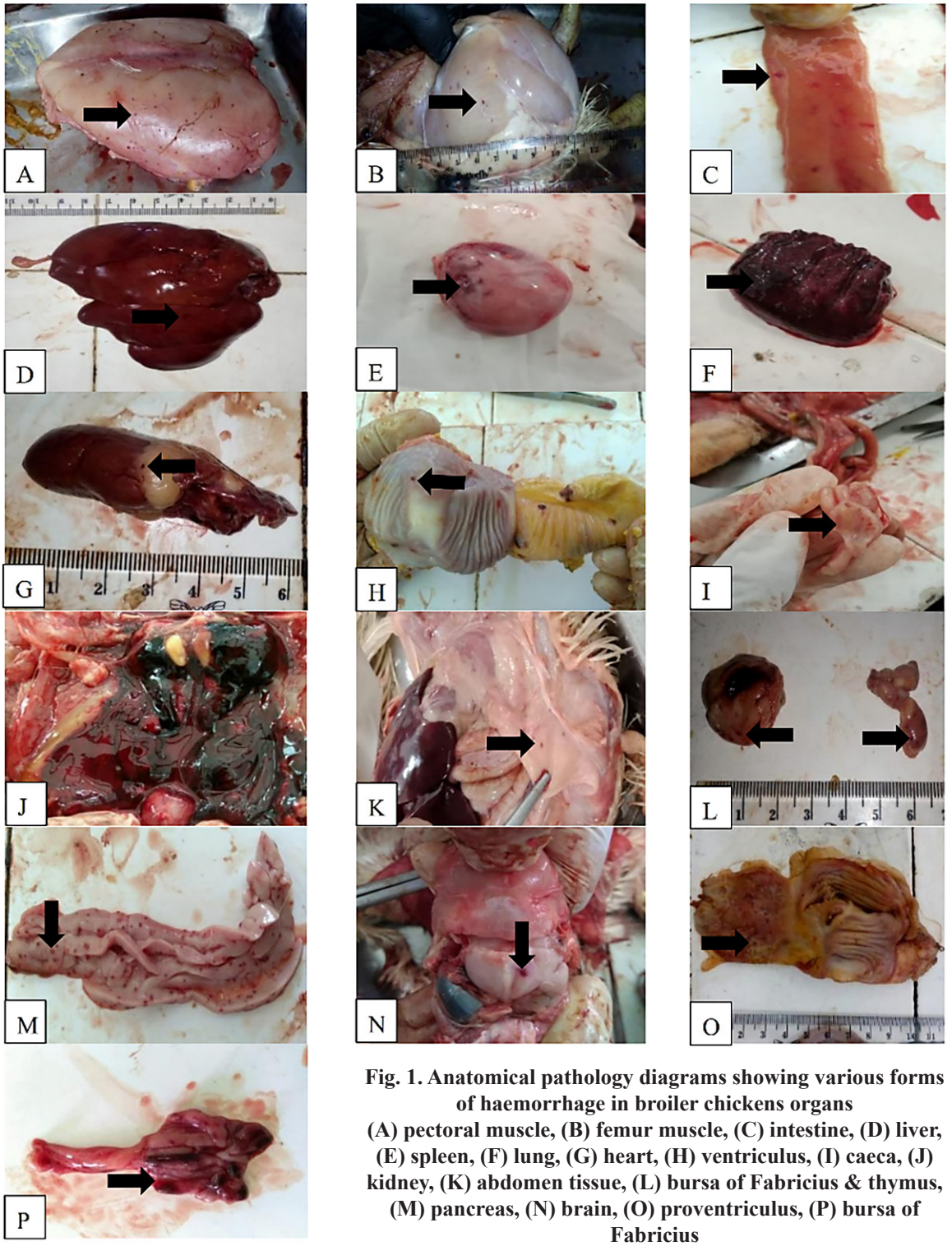

Fig. 1. Anatomical pathology diagrams showing various forms of haemorrhage in broiler chickens organs
(A) pectoral muscle, (B) femur muscle, (C) intestine, (D) liver, (E) spleen, (F) lung, (G) heart, (H) ventriculus, (I) caeca, (J) kidney, (K) abdomen tissue, (L) bursa of Fabricius & thymus, (M) pancreas, (N) brain, (O) proventriculus, (P) bursa of Fabricius

ples were then sliced using a microtome to a thickness of 5 µm and attached to a glass slide. Subsequently, the slides were stained using hematoxylin-eosin, examined using a microscope (Olympus CX23, Japan), and documented using a photomicrograph (Olympus DP20, Japan) (Kiernan, 2015).

Parasitological examination

The organs with lesions were ground with a mortar and mixed with saline solution to obtain a homogenous mixture. Subsequently, the drop of the mixture was poured on a glass slide, examined using a microscope with 40x magnification, and documented using a photomicrograph (Ballweber, 2001).

Results and Discussion

Anatomical pathology of chicken organ infected by Leucocytozoon sp

The anatomical pathology examination showed various forms of haemorrhage in the pectoral and femoral muscles, trachea, thymus, lungs, liver, heart, intestine, pancreas, spleen, abdominal tissue, ventriculus and proventriculus, kidney, caeca, bursa of Fabricius, as well as the brain (Figure 1). Furthermore, some organs, including the liver and spleen, were to be swollen due to inflammation. This study's gross anatomical finding is in line with previous reports where petechiae were observed on the pectoral muscle, as well as the pancreas (Lee et al., 2016), various forms of hemorrhage were observed on the femur and brachial muscle, thymus, kidney, skin, subcutaneous, lungs, intestine, and bursa of Fabriceus (Kaoud, 2017), haemorrhage was discovered on peritoneum cavity, perirenal, and subdural (Swayne, 2013), and swollen liver, as well as spleen were observed in penguins (Cannel et al., 2013).

The petechiae were present due to the destruction of endothelial cells, characterized by the pitted hemorrhage and a cleared boundary caused by a vascular leak. Most of the pathological changes resulting from severe *Leucocytozoon* infections are related to tissue damage caused by the inflammatory response to the megaloschizonts. A study by Gill and Paperna (2005) reported a link between the pathological process in anserine infections with *Leucocytozoon* and the development of megaloschizonts in several degenerative organs, including the spleen, liver, lungs, heart, and brain.

When *Simulium* sp and *Culicoides* sp flies suck the blood of poultry, the sporozoites in the insects' salivary glands automatically spread into the poultry blood circulation and enter the endothelial cells (kidneys, liver and lungs), as well as tissues and organs, including the heart, spleen, pancreas, thymus, muscles, intestines, trachea, ovaries, adrenal glands and brain (Valkiunas and Iezhova, 2023).

Subsequently, the sporozoites undergo a merogony process, during which they multiply through multiple divisions, known as schizogony, resulting in the formation of numerous merozoites that are released into the bloodstream. The release of these merozoites causes bleeding (petechiae) and damage to various organs and tissues (Ozmen and Haligur, 2005). The spread of hemorrhagic lesions to the kidneys and other organs occurs when merozoites are released from the megaloschizont. These merozoites, in turn, attack erythrocytes and develop into gametocytes (Suprihati, 2020). As the disease progresses, the poultry will experience hemorrhage and anemia, resulting in an increased mortality rate and decreased production (Kaoud, 2017).

Histopathological examination of chicken organs infected by Leucocytozoon

The histopathological examination showed the presence of schizont and megaloschizont in pectoral and femur muscles, trachea, lungs and liver (Figure 2). The megaloschizonts were shaped as described by Lee et al. (2016). They contained numerous basophilic schizonts in round, uniloc-ular structures surrounded by well-demarcated, eosinophilic-stained capsular walls of various sizes, which were present in either solitary or aggregated forms. These results are in line with the study by Pohuang et al. (2021), where schizonts and merozoites were observed in the muscles, bone marrow, brain, bursa of Fabriceus, heart, intestines, kidneys, liver, lungs, pancreas, spleen, thymus, and reproductive organs.

Damaged schizonts cause lymphocytic pneumonia, and in the heart, schizonts are found in the myocardium. The accumulation of mononuclear cells causes the epicardium to extend into the myocardium. Meanwhile, in the kidney, megaloschizonts cause hemorrhage and also trigger the depletion of zymogen and exocrine cells around the pancreas. Inflammatory or endothelial cells infected with *Leukocytozoon* develop into megaloschizont and release more merozoites (Zhao et al., 2014). The megaloschizont of *Leukositozoon* sp is described as a spherical structure containing several basophilic schizonts and surrounded by an eosinophilic capsule wall. This structure is often observed in various tissues and organs, including the liver, spleen, kidney, pancreas, heart, lungs, proventriculus, intestine, and brain (Swayne, 2013; Lee et al., 2016).

Parasitical examination

The results showed schizonts in three mashed organ samples, i.e., intestine, pectoral muscle, and lung, microscopically (Figure 3). The schizonts possess a host-derived capsular-like covering, which can vary in its level of visibility.

The native examination for leucocytozoonosis using mashed organs is less common compared to using blood samples, even though such results confirm leucocytozoonosis infection faster than histopathological results. The presence of large second-generation schizonts with thick capsules is pathognomonic and essential in diagnosing *L. caulleryi* infection (Figure 3). The size of these schizonts varies from 20.2 x 18.5 µm to 300 x 248 µm (Akiba, 1970). These capsules have thick and uniform walls. In chickens, there are minimal cellular responses against the second-generation schizonts enclosed in thick capsules (Nakamura, 2022). After the release of merozoites, the capsules rupture

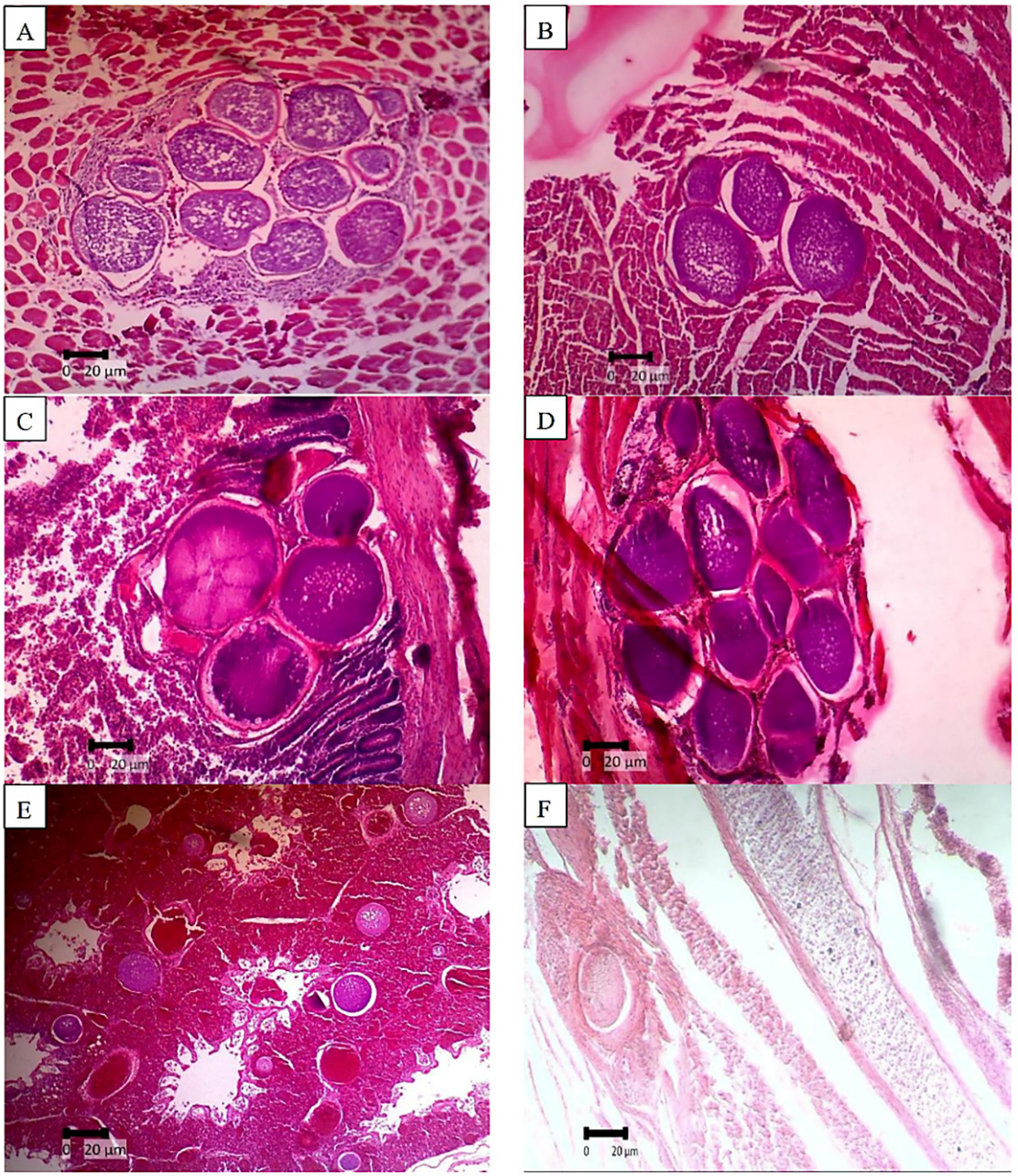

Fig. 2. Histopathology of broiler chicken organs showing schizonts and megaloschizonts in the (A) pectoral muscle, (B) femur muscle, (C) intestine, (D) heart, (E) lung and (F) trachea. (H&E, 40X, 10X)

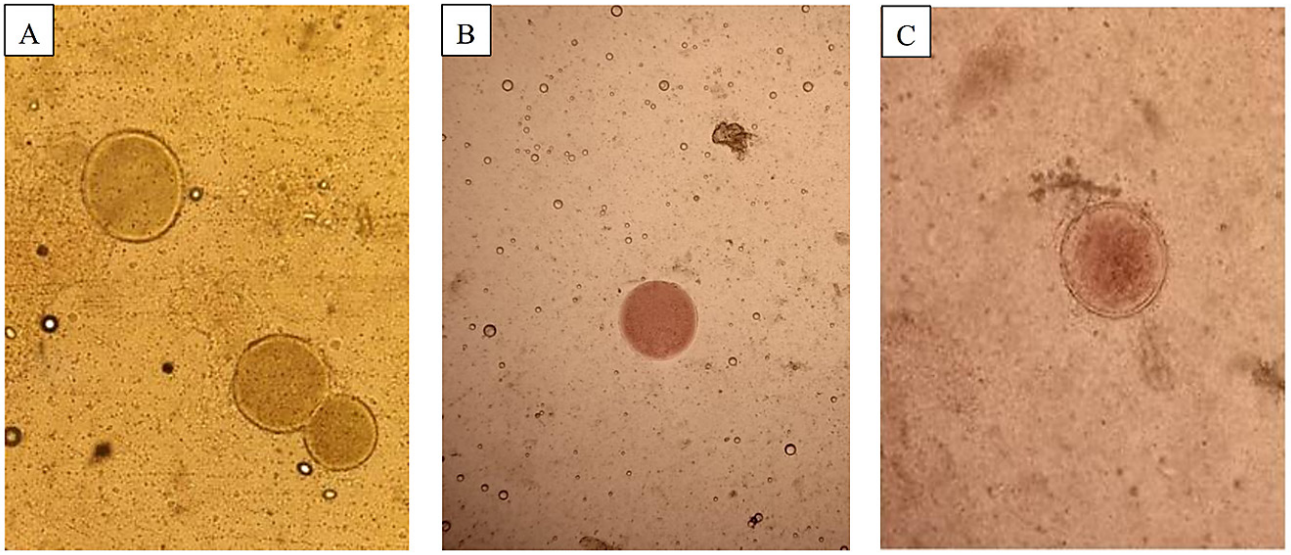

Fig. 3. Parasitological examination results showing the presence of schizonts in the (A) intestine, (B) pectoral muscle, and (C) lung. (40x)

and are surrounded by multinucleated giant cells, macrophages, lymphocytes, and heterophils (Lee et al., 2016). The capsular walls persist in the tissues after the release of merozoites before being phagocytized and absorbed (Valkiunas and Iezhova, 2023).

Conclusion

This study successfully diagnosed Leucocytozoonosis using anatomical pathology examination, histopathology, and parasitology methods, which revealed various hemorrhages, as well as schizont and megaloschizont forms, in almost all organs and tissues. Therefore, the combination of these three methods ensures the specificity of leucocytozoonosis diagnosis. However, treatment may only reduce parasitemia without eliminating the parasite; thus, the use of preventive medication and limiting insect vectors is recommended to help control the disease.

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