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REVIEW

Leucocytozoon caulleryi Infection in Chickens: Etiology, Pathology, and Diagnosis

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Abstract

In Southeast and East Asia, *Leucocytozoon caulleryi*, a protozoan of the order Haemosporida of the phylum Apicomplexa, causes anemia, hemorrhage, and mortality in young chickens, and anemia and hemorrhage with egg drop and soft-shelled eggs in layer hens. Leucocytozoonosis caused by *L. caulleryi* is a notifiable infectious disease in Japan because of the damage it causes to the poultry industry. During the summer, biting midges (*Culicoides arakawae*) transmit this protozoan to chickens. This review provides the etiology, pathology, and diagnosis of *L. caulleryi* infection in chickens.

Discipline: Animal Science
Additional key words: anemia, biting midge, egg drop, hemorrhage, protozoa

Introduction

Leucocytozoonosis in chickens is caused by *Leucocytozoon caulleryi*, *L. sabrazesi*, *L. andrewsi*, and *L. schoutedeni*, which belong to the order Haemosporida of the phylum Apicomplexa (van Wettere 2016). Of the four species, *L. caulleryi* is the most pathogenic, causing fatal hemorrhagic disease in young chickens and egg drop with soft-shelled eggs in laying hens. Leucocytozoonosis by *L. caulleryi* is one of the notifiable infectious diseases in the Japanese Act on Domestic Animal Infectious Diseases Control (Ministry of Justice, Japan 2009) because of the damage it inflicts on the poultry industry. Mathis & Leger (1909) discovered a protozoon that was not a malarial parasite in the peripheral blood of chickens in Vietnam and named it "*Leucocytozoon caulleryi*." Most species of *Leucocytozoon* are transmitted via the bite of various species of black flies (family Simuliidae). However, Akiba et al. (1958) discovered that *L. caulleryi* is transmitted by a biting midge (*Culicoides arakawae*). Bennett et al. (1965) proposed that "*Leucocytozoon caulleryi*" should be included in the new genus "*Akiba*." The genus name "*Akiba caulleryi*" was transiently used. However, Hsu et al. (1973) and Fallis et al. (1974) insisted that the genus "*Akiba*" was a subgenus of the genus "*Leucocytozoon*," and the term "*Leucocytozoon caulleryi*" has been used until now. Reviews on leucocytozoonosis by *L. caulleryi* in chickens are few. Thus, this review describes the etiology, pathology, and diagnosis of *L. caulleryi* infection in chickens. We believe that this review will be useful in diagnosing this disease for overseas researchers, especially in Southeast and East Asia.

1. Etiology

The life cycle of *L. caulleryi* has three stages: schizogony in the chicken's body, gametogony in the erythrocytes of the chicken, and sporogony in the biting midge (*C. arakawae*) (Akiba 1970; Morii 1978, 1992). Schizogony is divided into first and second generations (Akiba 1970). Sporozoites invade the vascular endothelial cells of the various organs and tissues of chickens. Merozoites are released from schizonts and invade

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additional vascular endothelial cells of the systemic organs in the form of second-generation schizonts 5-6 days after infection by sporozoites. Mature second-generation schizont can reach sizes of up to about 300 μm (Akiba 1970). These are referred to as "megaloschizont" (Goto et al. 1966, Lee et al. 2016, Miura et al. 1973) and are most pathogenic to chickens. Second-generation schizonts cause pressure atrophy of the surrounding tissues at the site of parasitic invasion, resulting in vascular injury due to endothelial tissue parasitism, hemorrhage, edema, and granulomatous reactions against the capsular walls of the ruptured schizonts (Akiba 1970). The merozoites within the second-generation schizonts are released, which parasitize erythrocytes in the circulating blood 14 days after the inoculation of sporozoites. Following their invasion into the erythrocytes, the merozoites undergo gametogony. That is, merozoites grow and differentiate to gametocytes. There are five stages of gametogony (Akiba 1970): stage I, the release of free merozoites from the schizonts; stage II, the appearance of gametocytes, which are the same size as merozoites, in the cytoplasm of the erythrocytes; stage III, the enlargement of the gametocytes; stage IV, the presence of the host cell nuclei in large gametocytes; and stage V: the absence of the host cell nuclei in large gametocytes. Macrogametocytes (female) and microgametocytes (male) can be differentiated in stages IV and V. The gametocytes deform the erythrocytes and cause them to decay over time at 18 d after infection by sporozoites. Macrogametocytes and microgametocytes are taken into the midguts of biting midges when they feed on an infected chicken's blood. The macrogametes and microgametes undergo fertilization to form zygotes and subsequently ookinetes in the midguts of biting midges. Ookinetes invade the interspaces of the midgut walls, move to the serosa of the midgut, and develop into oocysts. Several dozen sporozoites develop into oocysts. After oocysts burst, the sporozoites are released into the body cavity and move to the salivary glands of the biting midges. The sporozoites enter the chickens when they are bitten by the infected midges.

2. Epidemiology, clinical symptoms, hematology, and serology

Leucocytozoon caulleryi infections have been reported in Japan (Akiba et al. 1958, Fukuda et al. 1987, Goto et al. 1966, Jin et al. 1976, Miura et al. 1973, Nakamura et al. 1997, Okazaki et al. 1967, Yamada et al. 1981), South Korea (Lee et al. 2014, Lee et al. 2016), Taiwan (Pan 1963, Yu et al. 2000), China (Zhao et al. 2016), Indonesia (Suprihati & Yuniarti 2017), Thailand (Morimoto & Shiku 2003), Malaysia (Gimba et al. 2014), Myanmar (Griffiths 1963), Philippines (Manuel 1969), India (Sawale et al. 2018), Bangladesh (Nath et al. 2014), and other countries. In East Asian countries, such as Japan and South Korea, the onset of this infection usually occurs during the summer when biting midges (*C. arakawae*) are active. This disease can occur throughout Japan, although it is not usually found in areas with cold climate, such as Hokkaido, North Japan. Paddy fields and/or ponds are suitable habitats for biting midges and are often found near poultry houses (Fukuda et al. 1987, Jin et al. 1976, Yamada et al. 1981). In tropical countries, the disease is prevalent throughout the year (Suprihati & Yuniarti 2017).

The clinical symptoms of *L. caulleryi* infection are anemia; egg laying abnormalities, such as decreased and abnormal (soft-shelled eggs) egg production; and green diarrhea in layer hens (Jin et al. 1976, Nakamura et al. 1997, Sawale et al. 2018, Yamada et al. 1981). The disease is frequently seen in laying hens, particularly in recent years, because of strict regulations on the use of antiprotozoal agents in laying hens. Fatal infections with systemic hemorrhages and schizont parasitism are sometimes observed in growing chicks (Fukuda et al. 1987, Goto et al. 1966, Lee et al. 2014). The mortality rates caused by this disease varied from approximately 10 to 60% in young chickens (Fukuda et al. 1987, Goto et al. 1966, Okazaki et al. 1967) and less than several percentages in adult chickens (Jin et al. 1976, Lee et al. 2016, Miura et al. 1973, Okazaki et al. 1967).

Hematologically, decreases in the number of mature erythrocytes and increases in the number of immature erythrocytes (polychromatophilic erythrocytes) are seen in infected hens and chicks with parasitemia (Jin et al. 1976, Okazaki et al. 1967). Jin et al. (1976) reported that the mean erythrocyte number and mean hematocrit value of uninfected chickens were 276×10^4/mL and 29%, respectively, and those of infected chickens were 186×10^4/mL and 21%, respectively. The presence of gametocytes in the erythrocytes in blood smears is an important diagnostic factor for this disease (Fukuda et al. 1987, Yamada et al. 1981). Gametocyte stages II and V are associated with anemia in the peripheral blood of infected chickens (Fukuda et al. 1987, Jin et al. 1976, Pan 1963, Yamada et al. 1981).

In Giemsa-stained blood smears, stage V macrogametocytes appear basophilic, whereas stage V microgametocytes appear eosinophilic (Fig. 1) (Isobe 2010). This difference in the staining characteristics of macrogametocytes and microgametocytes is useful for the differential diagnosis of gametocytes. Under transmission electron microscopy, macrogametocytes

have been seen to contain more ribosomes than microgametocytes (Morii et al. 1981). The ribosomes contain a large amount of ribonucleic acid, which is basophilic (Fujita & Fujita 1981). Macrogametocytes, which have a high density of ribosomes, appear to exhibit more basophilic properties than microgametocytes. The blood smear findings of the gametocytes of *L. caulleryi* showed that they lacked pigments and could therefore be differentiated from those of avian malaria, which contain malarial pigments. Additionally, *L. caulleryi*-infected erythrocytes never exhibit intra-erythrocytic schizonts observed in the blood smears of chickens infected with avian malaria.

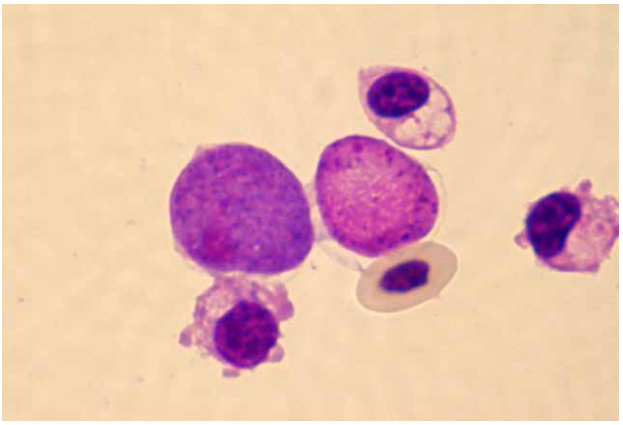

Fig. 1. Gametocytes in blood smear
Macrogametocyte (left) and microgametocyte (right). Field case of layer. Giemsa staining.

For the serological tests of leucocytozoonosis, serum antibody and antigen are detected in the sera of infected chickens using agar gel precipitation (AGP) (Morii 1972, 1992). The serum antigen is found at 10-15 days and the serum antibody at 17-98 days after the inoculation of sporozoites. Additionally, counterimmunoelectrophoresis (Fujisaki et al. 1980), immunofluorescence (Fujisaki et al. 1981, Isobe & Akiba 1982), an enzyme-linked immunosorbent assay (Isobe & Suzuki 1986), and a latex agglutination test (Ito & Gotanda 2005) have been developed. However, of these tests, the AGP for detecting serum antibody is the only commercially available test (Scientific Feed Laboratory Co., Ltd., Tokyo, Japan).

Pathology

1. Gross pathology

Adult chickens infected with leucocytozoonosis have pale combs, wattles, and faces (Jin et al. 1976, Manuel et al. 1969, Yamada et al. 1981); splenomegaly (Nakamura et al. 1997, Okazaki et al. 1967, Sawale et al. 2018, Yamada et al. 1981); ovarian follicle atrophy; and edema of the oviduct (Lee et al. 2016, Nakamura et al. 1997). On rare occasions, some affected hens show severe hepatic hemorrhages like those associated with fatty liver hemorrhagic syndrome (Lee et al. 2016). Hemorrhages of the subcutis, muscle, trachea, liver, kidney, lung, spleen, pancreas, thymus, and bursa of Fabricius can be found in infected growing chicks (Fukuda et al. 1987, Goto et al. 1966, Okazaki et al. 1967). Leucocytozoonosis can be observed as diffuse petechial hemorrhages in the muscles of broilers at processing (Hangui 2014).

2. Histopathology

Histologically, the presence of second-generation schizonts in the endothelial cells of blood vessels, the formation of thrombus, hemorrhages, and reactive inflammation against schizonts in the systemic organs are characteristic of *L. caulleryi* infection (Goto et al. 1966, Lee et al. 2016, Miura et al. 1973, Nakamura et al. 1997). Hemosiderin deposition in the livers and spleen, which appears to result from the destruction of erythrocytes by *L. caulleryi* gametocytes, has also been observed (Goto et al. 1966). There are two schizont stages, resulting in first- and second-generation schizonts. Generally, large second-generation schizonts (megaloschizonts) are found in the histological sections of field cases infected with *L. caulleryi* (Fukuda et al. 1987, Goto et al. 1966, Hangui 2014, Lee et al. 2014, Lee et al. 2016, Miura et al. 1973, Nakamura et al. 1997).

Okazaki et al. (1967) detected second-generation schizonts and lesions in the ovaries, oviduct, and muscles (100%); lungs (72%); kidneys (67%); heart (60%); and liver (58%) of 59 layer hens, as well as in the lungs, liver, kidneys, and bursa of Fabricius (100%); duodenum (80%); and spleen (67%) of 7 broiler chicks. Occasionally, second-generation schizonts, merozoites, and granulomatous inflammation can be observed following the release of merozoites in the cerebrum and cerebellum (Fukuda et al. 1987, Lee et al. 2014, Miura et al. 1973).

Greenish diarrhea is often noted in hemorrhagic diseases, such as Newcastle disease (Miller & Koch 2013). Intestinal hemorrhages are seen in chickens infected with *L. caulleryi* (Goto et al. 1966, Okazaki et al. 1967). Greenish diarrhea (Goto et al. 1966, Jin et al. 1976, Manuel 1969, Okazaki et al. 1967, Yamada et al. 1981) may be attributed to hemorrhages in the digestive tract of affected chickens.

The pathogenesis of egg drop and soft-shelled eggs in leucocytozoonosis was pathologically evaluated in field cases (Nakamura et al. 1997) and experimental cases (Nakamura et al. 2001); it was found that the *L. caulleryi* schizont development, granulomatous lesions, and cellular infiltration in the ovaries and oviducts were

associated with layer hens showing egg drop with eggshell abnormalities (soft-shelled eggs) (Nakamura et al. 1997). The degeneration and atrophy of follicles with granulomatous lesions against *L. caulleryi* second-generation schizonts in the ovaries and the reduction of secretory glands and edema with granulomatous lesions against *L. caulleryi* schizonts in the uterus of the oviduct were observed. The degeneration and atrophy of follicles in the ovaries appeared to be a change linked to reduced egg production, and the lesions in the uterus of the oviducts that secrete the eggshell of the egg may explain the soft-shelled eggs in layer hens infected with *L. caulleryi*. Nakamura et al. (2001) inoculated specific pathogen-free hens with *L. caulleryi* and confirmed its pathogenicity. Histologically, many large second-generation schizonts were found in the ovaries (Fig. 2) and oviducts (Fig. 3). The schizonts caused the compression and atrophy of the surrounding tissues. Following the release of merozoites, marked granulomatous inflammation occurred in response to the degenerated capsules of second-generation schizonts. Of the oviduct, the uterus was the most severely affected. These changes were similar to the ovary and oviduct lesions seen in field cases. From this, it was proved that *L. caulleryi* has an affinity for the ovaries and oviducts, particularly the uterus of the oviduct, in hens. These changes in the ovaries and oviducts explain the clinical signs of egg drop and soft-shelled eggs in infected hens.

Death begins to occur among infected young chickens at 13-16 days after inoculation with sporozoites because of sudden hemorrhages (Akiba 1970). The death of chickens by *L. caulleryi* infection appears to be caused by anemia, hemorrhages, and embolism induced by second-generation schizonts and gametocytes in blood vessels (Goto et al. 1966). If chickens survive fatal infection, they often subsequently suffer from anemia (Akiba 1970). It is thought that the anemia results from the hemorrhages caused by the schizont parasitizing the vascular endothelium and the subsequent destruction of erythrocytes by gametogony within them (Akiba 1970).

"Hepatic schizont," early schizont in the cytoplasm of the hepatocytes, has been reported for *L. smith* infection in turkeys (Newberne 1955) and *L. simondi* infection in ducks (Newberne 1957). Goto et al. (1966) describes "hepatic schizont" in the cytoplasm of the hepatocytes in chicks infected with *L. caulleryi*. There have not been any other reports on "hepatic schizont" in the *L. caulleryi* infection in chickens. This should be evaluated further in the future. Schizonts can be observed in the brain and meninges (Akiba et al. 1970, Fukuda et al. 1987, Lee et al. 2014) and bone marrow (Lee et al. 2014) of young chickens fatally affected by leucocytozoonosis. The wide distribution of schizonts and severe lesions seen in chicks may be due to their increased susceptibility to *L. caulleryi*.

Large second-generation schizonts with thick capsules are pathognomonic and crucial in the diagnosis of *L. caulleryi* infection (Fig. 4). The size of the schizont ranges from 20.2 × 18.5 μm to 300 × 248 μm (Akiba 1970). The capsular walls are thick, homogeneous, and eosinophilic. The morphogenesis of the capsular walls of *L. caulleryi* is not well understood. Newberne (1957) described the capsular walls of *L. simondi* in ducks as reticular fibers originating from the host tissue around the schizonts.

There are few cellular reactions against the

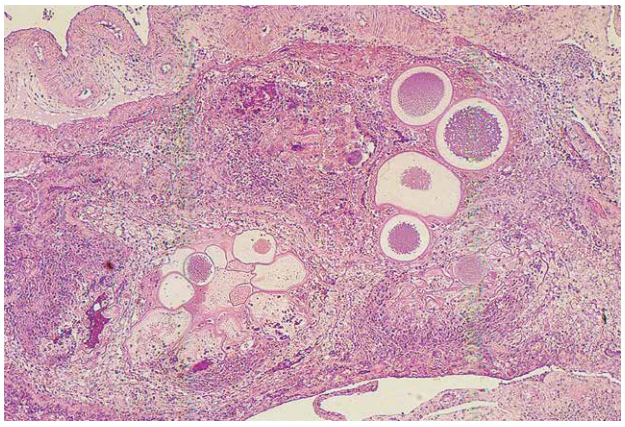

Fig. 2. Second-generation schizonts in the ovary
Many schizonts parasitize the stroma of the ovary. Merozoite-liberated schizonts surrounded by macrophages. Field case of layer. Hematoxylin and eosin staining.

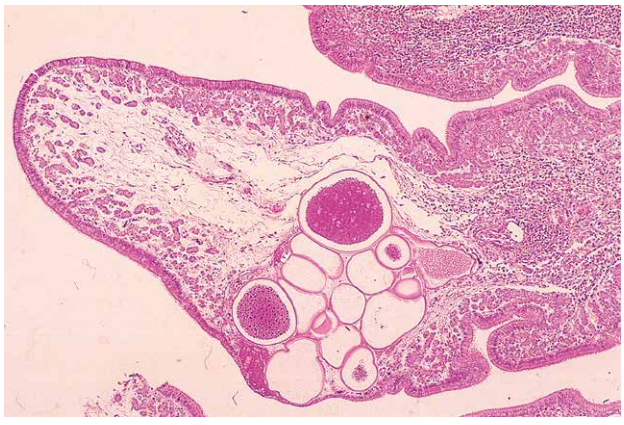

Fig. 3. Second-generation schizonts in the uterus of the oviduct
Loss of glands, edematous loosening, and schizonts parasitizing in the lamina propria of the uterus of the oviduct. Field case of layer. Hematoxylin and eosin staining.

second-generation schizonts enclosed by thick capsules in chickens. The capsules rupture after the release of merozoites and are surrounded by multinucleated giant cells and macrophages, lymphocytes, and heterophils (Goto et al. 1966, Lee et al. 2016, Miura et al. 1973, Nakamura et al. 1997). The capsular walls remain in the tissues after the release of merozoites, before being phagocyted and absorbed.

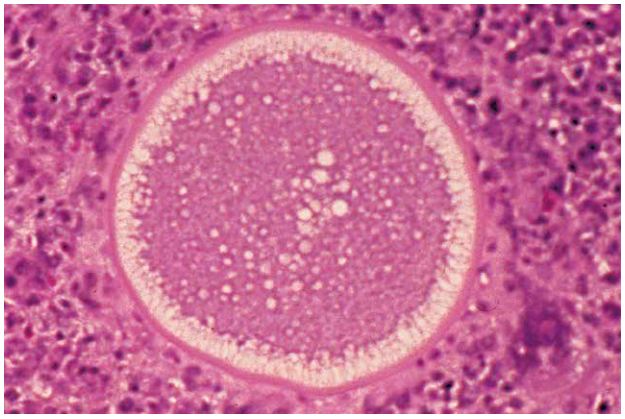

Fig. 4. Second-generation schizont in the lung
The schizont capsule wall is thick. Many merozoites are present within the schizont. Foreign body giant cell is seen around the schizont (lower right). Field case of layer. Hematoxylin and eosin staining.

Granulomatous inflammation with lymphocytic infiltration against second-generation schizont capsules occurs in the peripheral nerves (Fig. 5) and brains (Fig. 6) of affected chickens (Fukuda et al. 1987, Nakamura et al. 1997). Since the inflammatory response remains even after the absorption of the schizont capsules, the lymphocytic infiltration of the peripheral nerve may be confused with the changes seen in Marek's disease. However, more detailed histological examination will find the capsules.

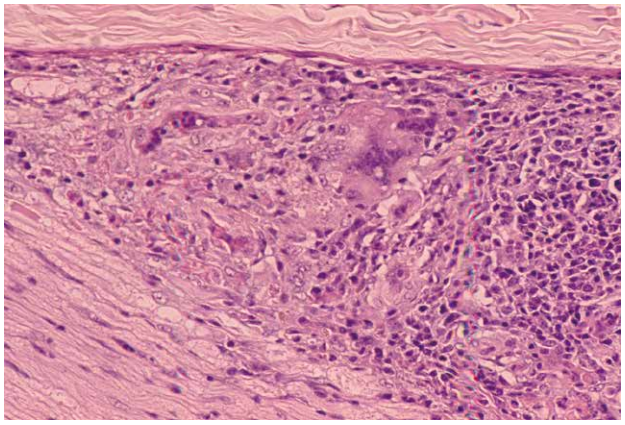

Fig. 5. Granuloma with lymphocytic infiltration in the peripheral nerve
Field case of layer. Hematoxylin and eosin staining.

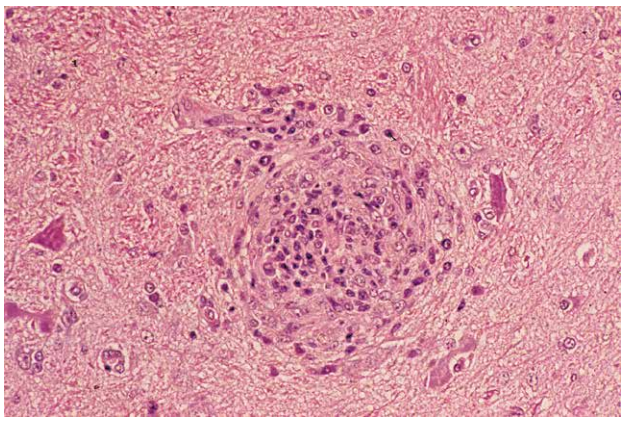

Fig. 6. Granuloma with lymphocytes in the cerebrum
Field case of layer. Hematoxylin and eosin staining.

3. Molecular pathology

Polymerase chain reaction (PCR) and sequencing analysis of PCR products with formalin-fixed, paraffin-embedded (FFPE) tissues (Lee et al. 2016) and fresh organs (Lee et al. 2014) with specific lesions containing *L. caulleryi* megaloschizonts are performed for diagnosis and epidemiology. Lee et al. (2016) suggested that the *L. caulleryi* strain isolated in South Korea was closely related to the strains found in Japan and Malaysia. In undiagnosed cases, in which no *L. caulleryi* parasites were observed in the histological sections, PCR of FFPE tissues and sequencing analysis of PCR products may be useful. These techniques are also useful in the retrospective studies of past cases. Suprihati & Yuniarti (2017) diagnosed *L. caulleryi* and *Plasmodium* sp. infections based on the PCR analysis of blood samples from broilers in Indonesia. Zhao et al. (2016) used PCR and microscopic examination to reveal that *L. sabrazesi* was more prevalent than *L. caulleryi* in free-range and backyard chickens in Southern China. They also noted that PCR assays were more sensitive than microscopic examination for detecting *L. sabrazesi* infection. The molecular investigations of *L. caulleryi* are mainly conducted using the primers to amplify the mitochondrial cytochrome genes (Lee et al. 2014, Lee et al. 2016, Zhao et al. 2016, Suprihati & Yuniarti 2017). Especially, the nested PCR method by Hellgren et al. (2004), using two pairs of primers against the mitochondrial genes, is often used (Lee et al. 2016, Suprihati & Yuniarti 2017). Besides mitochondrial genomes, Imura et al. (2014) analyzed the genome of the apicoplast, a vestigial plastid of *L. caulleryi*. They expected that the molecular analysis of the apicoplast contributed to the improvement of the accuracy of both the phylogenetic analysis and genetic typing of apicomplexan parasites, including *Leucocytozoon*.

Diagnosis

Leucocytozoonosis by *L. caulleryi* in chickens is mainly diagnosed by clinical findings, hematology, and histopathology. *Leucocytozoon caulleryi* can be differentiated from other chicken *Leucocytozoon* species by the morphology of the gametocytes and host cell in blood smears. The genera *Leucocytozoon* and *Plasmodium* belong to the order Haemosporida, and both parasitize the erythrocytes of chickens (Bermudez 2013). Both schizogony and gametogony with malarial pigments can be observed in the erythrocytes of chickens infected with *Plasmodium*, whereas gametogony without pigments is only found in the erythrocytes in *L. caulleryi* infection. Hyperplasia of macrophages with malarial pigment of macrophages is seen in chickens infected with *Plasmodium juxtanucleare* (Hidaka et al. 2015) or *P. gallinarum* (Isobe et al. 2006). Thus, the differential diagnosis of leucocytozoonosis and avian malaria may be easily performed based on blood smears and the histological findings of affected chickens. Furthermore, molecular investigation is an effective method in differentiating other hemosporidian infections.

Conclusions

Leucocytozoon caulleryi infection occurs during the summer season. The clinical characteristics of this infection include anemia, egg drop, and soft-shelled eggs in laying hens; fatal systemic hemorrhages in chicks; and the detection of gametocytes in blood smears. Macroscopically, petechial hemorrhages can be found in various organs, such as the liver, kidneys, lungs, spleen, pancreas, thymus, bursa of Fabricius, and muscles. Histologically, second-generation schizonts in the vascular endothelial cells with hemorrhages and granulomatous lesions are pathognomonic. Molecular investigation by PCR and sequencing is also useful for more accurate diagnosis and epidemiology.

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