Original Article

Development of A Confident, Reliable Diagnostic Method for the Viral and Avian Blood Parasites Co-infection Associated with Hemorrhagic Syndrome in Broiler Chickens

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Abstract

Egyptian poultry farms are often impacted by many avian diseases, resulting in significant annual losses due to single or combined infections. This study sought to implement a more integrated approach for diagnosing etiological agents linked to hemorrhagic syndrome in broiler chickens, utilizing a newly developed set of primers to detect and identify four prevalent avian haemosporidians. These methodologies serve as a framework for the routine identification and monitoring of field avian pathogens, marking the initial effort for early prevention and control. Real-time PCR, blood smear, hematological analysis, and histopathological examination were employed for the detection and identification of several etiological agents. Our qPCR results identified co-infections in affected flocks including Newcastle disease virus genotype II (NDV-GVII) and *Leucocytozoon* species (*L. sabrazesi* and *L. caulleryi*), alongside other viruses such as avian influenza virus (AIV) (H9), infectious bursal disease virus (IBDV), chicken infectious anemia virus (CIAV), and blood protozoa (*Haemoproteus* spp., *Plasmodium* spp.), occurring as triple infections or more in Sharqia Province. This study presents the first documentation of *L. sabaresizi* in broiler chicken populations in Egypt. Two species of *Leucocytozoon* (*L. sabrazesi* and *L. caulleryi*), and *Plasmodium gallinaceum* were identified based on the morphological characteristics of gametocytes in blood smears. A notable reduction in hematological markers. The total leucocytic count (TLC) was markedly reduced in all flocks, particularly in co-infected CIAV flocks (1 and 3), compared to flock no. 2. Histopathological analysis revealed prominent megaloschizonts, indicative of *Leucocytozoon*, accompanied by significant pathology changes resulting from mixed infections. Finally, Egyptian poultry flocks are often impacted by mixed pathogens. So, the simultaneous detection and differential diagnosis of these co-infections offers a crucial reference for poultry producers to implement prompt and effective disease management measures.

Keywords: Hemorrhagic syndrome, co-infection, *Leucocytozoon sabrazesi*, NDV-GVII, Broiler chickens

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1. Introduction

The Egyptian poultry industry is one of the significant sectors of Egyptian agricultural production, contributing 1125 million tons of total poultry meat (FAO, 2017). Small-scale Egyptian intensive production systems exhibit insufficient feed, water, veterinary services, recurrent disease outbreaks, adverse weather conditions, and restricted bio-security measures (Abu Hatab et al., 2021). Egyptian poultry farms are repeatedly impacted by many avian diseases, whether through singular or mixed infections, leading to substantial annual losses (Megahed et al., 2020).

The anemic diseases of chickens may result from bone marrow aplasia, hemorrhages, and atrophy of the lymphoid organs, according to several researchers (Peckham, 1984). Anemia involves multiple etiological factors, including acute bleeding (hemorrhage), erythrocyte destruction by blood parasites or toxins, and diminished erythrocyte synthesis in the bone marrow (Campbell, 2014). Thrombocytopenia characterized by diminished thrombocyte levels may occur after septicemia in avian species (D'Aloia et al., 1994). The most endemic blood parasites in chickens are *Plasmodium, Haemoproteus, and Leucocytozoon* (Zhang et al., 2014).

Leucocytozoonosis contributes to significant economic losses in the poultry sector, including weight reduction and anemia (McDougald et al.,

2020) *Leucocytozoon caulleryi* and *Leucocytozoon sabrazesi* are the two common species causing leucocytozoonosis in chickens (Zhao et al., 2016). Recent reports indicate that Egyptian commercial broilers are infected with *Leucocytozoon caulleryi* (Elbestawy et al., 2021; Abou El-Azm et al., 2022).

Immunosuppressive viruses, including Infectious Bursal Disease (IBDV), Chicken Infectious Anemia (CIAV), and Inclusion Body Hepatitis (Fowl Adenovirus "FAdV") induce immune cell depletion and mortalities (Dhama et al., 2002).

Detecting leucocytozoonosis based solely on clinically observed pathological lesions is challenging, as these lesions may be confused with other lesions accompanied by hemorrhagic syndrome such as in IBDV, CIAV, Avian Influenza virus (AIV), Newcastle Disease virus (NDV), and Fowl Adenovirus (FAdV) (Abou El-Azm et al., 2022). The field observations, conventional parasitological, and virologic diagnostic tests are insufficient for proper detection and confirmation (Zhao et al., 2016; Abou El-Azm et al., 2022). Molecular diagnosis enables precise and rapid identification of infections (Laamiri et al., 2018), hence improving the implementation of timely control measures and subsequently mitigating economic losses. From this point, we designed genus-specific primers capable of efficiently amplifying the target region of the four predominant avian haemosporidian DNAs individually, without cross-amplifying DNA from other co-infecting parasites in the collected samples.

This study sought to implement integrated resilience-based methodologies for identifying causative agents associated with anemia and hemorrhage in muscles and internal organs. Additionally, the study aimed to develop a molecular diagnostic method that enables the precise and rapid identification and separation of the four prevalent haemosporidian parasites in chickens in Egypt.

2. Material and Methods

This study was approved by the Zagazig University- Institutional Animal Care and Use Committee (ZU-IACUC committee) in Egypt's guidelines collection of samples from non-living animals (approval No: ZU-IACUC/2/F/39/2024).

2.1. Case History and Necropsy Findings

Three broiler poultry farms were submitted to necropsy at the Avian and Rabbit Medicine Department, Faculty of Veterinary Medicine, Zagazig University. They were 22-28 days old and were sourced from various discrete in the Sharqia government during the summer season. All of them were reared in a semi-intensive production system with stocking density (3000-5000 birds), and mortality rate (3-10%) at the time of sampling. Infected birds showed paleness, weight reduction, ruffled feathers, and subcutaneous hemorrhaging. The pathological lesions were examined and assigned for severity based on a lesion score ranging from 0 to 3, where a score of 0 means no lesions, a score of 1 indicates mild or slight lesions, a score of 2 indicates moderate lesions and a score of 3 indicates severe lesions (Jirjis et al., 2004) (Table 1).

2.2. Collection of Samples

2.2.1. Collection and Processing of Tissue Samples

Tissue samples were collected from 27 necropsied broiler chickens (7, 12, & 8 birds per flocks 1, 2, & 3, respectively), including the liver, heart, lung, spleen, thymus, muscles, bursa, kidney, junction of the proventriculus, and gizzard. One-half of these samples were homogenized by adding 20 mg of solid tissue with 300 μl PBS in a 2 ml tube for molecular diagnosis (de Francisco et al., 2023), while the other half of the samples were used for histopathological examination.

2.2.2. Collection of Blood Samples

Blood samples were collected from diseased chickens (7 live birds as 2, 1, & 4 birds per flocks 1, 2, & 3 respectively) with and without anticoagulant (1 mg of dipotassium salt of EDTA per 1 ml blood), for blood smear and hematological assessment, respectively.

2.3. Molecular Analysis

2.3.1. RNA and DNA Extraction

Viral RNA was extracted from pooled tissue sample supernatants (5-7 bird tissues per flock), for detection of NDV, AIV, and IBDV using QIAamp Viral RNA extraction kit. Concurrently, DNA from other viruses and blood protozoa was extracted using a DNA extraction kit (Qiagen, USA, Catalog No. 52904), for the detection of FAdV,

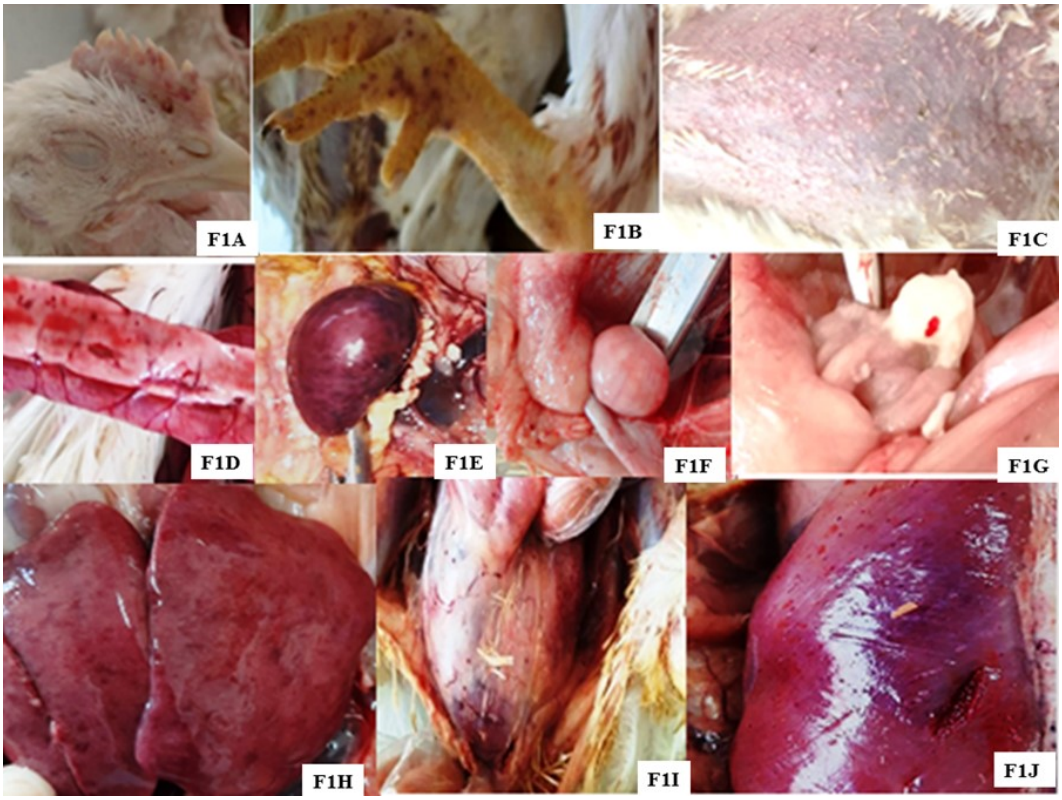

Figure 1: Post-mortem lesions of the flock no 1. (A) Paleness with petechiae in comb and wattle. (B) Petechial hemorrhage on the shank in the shape of the nodule. (C) Nodular skin appearance. (D) Hemorrhage on the pancreas. (E) Hemorrhage on the spleen. (G) Caseated bursa. (H) Petechial hemorrhage on the liver. (I) Erythema, and petechial hemorrhage (circular spots) on the abdominal cavity. (J) Severe hemorrhage on the keel muscle.

CIAV, and four distinct blood protozoa following the manufacturer's protocol outlined in the (QI-Aamp Min Elute Virus Spin Handbook,3rd edition, April 2010).

2.3.2. Oligonucleotide Primers

All used primers in this study listed in Table 2 were supplied from Metabion (Germany). Our oligonucleotide primers are designed to be species-specific and to produce PCR products with a minimum of 150 bp and a maximum of 223. Primers of *subtilisin-like proprotein protease* (*SPP*) and *cytochrome b* (*Cytb*) were designed using Primer3 and Fast PCR software. These primers were refined for specificity and sensitivity using "touchdown PCR" and were validated through experimental testing. The specificity of forward and reverse primer sequences, melting temperature, and the expected amplicon size are listed in Table 2.

2.3.3. Conditions for Real-Time PCR (qPCR)

Condition for NDV, NDV-GVII, AIV H5, AIV H9, and IBDV RT-PCR was performed using GoTaq® 1-step RT-PCR kit purchased from Promega®, USA). The reaction was performed in the Animal Health Research Institute, Zagazig Branch, Biotechnology Unit, using a real-time PCR system (Applied Biosystems, Foster City, CA, USA). Each PCR reaction volume (25 μl), comprised of 12.5 μl GoTaq qPCR master mix (2×), 2ul Forward primer, 2 ul Reverse primer, 0.5 μl GoScript™ RT mix, 5 μl RNA template, 0.5 μl CXR Reference dye, and Nuclease-Free water Up to 25 μl.

Condition for CIAV, FAdV, and Avian Haemosporidian The PCR reaction volume (25 ul) comprised 14.5 μl of master mix (GoScript™ RT mix), 5 μl of the extracted DNA as a template, 1μl each of forward and reverse primers, 0.5 μl of CXR Reference dye and deionized water up to 25 ul master mix content.

Thermal Profile of qPCR Protocol Reverse transcription was performed for one cycle at 37°C for 15 min for all detected agents as follows: initiate with primary denaturation for one cycle at 95°C for 10 minutes, followed by 40 cycles of secondary denaturation at 95°C for 10 minutes and annealing at specific melting temperatures (Tm) ranged

Table 1: Post-mortem lesions in investigated flocks.

Organs	Lesion	Investigated flocks[a]		
		Flock 1 [b,e]	**Flock 2**	**Flock 3** [c,d,e]
Comb & wattle	Paleness	++ with petechiae	**++++**	**++++**
Heart	Paleness	++++ (5/7)	++++ (10/12)	++++ (4/8)
	Congestion	++	-	-
	Hemorrhage	-	-	++ (2/8)
	Necrosis	-	-	++
Liver	Hemorrhage	++++ (4/7)	-	-
	Petechiae	-	-	++ (1/8)
	Subcapsular hemorrhage	-	-	++++ (5/8)
	Congestion	6/7 ++++	-	
	Peripheral infarction	-	-	++ (2/8)
	Paleness	-	++++ (10/12)	-
Brain	Congestion	-	++	++++ (5/8)
	Hemorrhage	-	-	++ (1/8)
Shank	External hemorrhage (as nodules)	++++	-	++++
	Petechiae inside the ligament	++++	-	-
Rectum	Hemorrhage	-	-	+++ (4/8)
Skin & SC tissues	Hemorrhage as nodules	++++		+++++
Pancrease	Hemorrhage	++++ (7/7)	-	++ (1/8)
	Necrosis	-	-	++ (3/8)
Lung	Congestion	++	-	++++
	Paleness	-	++++	-
	Hemorrhage	-	-	++++
Bursa of Fabricius	Hemorrhage	-	-	-
	Atrophy	-	++++	+++ (3/8)
	Enlarged	++++	-	++++ (4/8)
	Casiated	++	-	-
Spleen	Hemorrhage	+++	++++ (2/ 12)	++ (3/8)
	Atrophy	-	+++	-
	Congestion	++[1]	-	-
	Pale	-	++++	++++ (2/8)
	Mottled	++++ (4/7)	-	-
Thymus	Atrophy	-	++++ (10/12)	-
	Congestion	-	-	++++ (1/8)
	Hemorrhage	+++	+++	++++ (6/8)
Proventricular-ventricular junction	Hemorrhage at papillae	+++	-	++++ (2/8)
Thigh & breast muscle	Paleness	-	+++ (10/12)	++ (1/8)
	Congestion	++ ++ (1/7)	++++(2/12)	++ (1/8)
	Hemorrhage as nodules	++++ (4/7)	++(2/12)	++++ (4/8)
Kidneys	Hemorrhage	++++ (2/7)	++++ (2/12)	++++ (5/8)
	Necrosis	-	-	++ (1/8)
	Enlarged & pale	++++ (3/7)	+++ (10/12)	+++ (2/8)
Total= 15	= 42	=21	=16	= 29

[a] Abbreviations: - = not detected ++ = mild +++ = moderate ++++= severe, (positive/tested).
[b] Circular hemorrhage spots inside the buccal cavity in flock no 1.
[c] Hemorrhage on the inner side of keel-bone in flock no 3 (4/8).
[d] Circular hemorrhagic spots in tissues around the neck, thymus, and trachea, degenerative changes at the base of the heart in flock no 3.
[e] Sever congestion in the trachea and surrounding tissues in flocks no 1&3.

from 58-60°C for 30 seconds (Table 2), concluding with extension at 72°C for 10 seconds.

The control positive was a field strain obtained from the Reference Laboratory for Veterinary Quality Control on Poultry Production at the Animal Health Research Institute, whereas the negative control was a PCR master mix without DNA template.

2.3.4. Hematological parameters

Erythrocyte count (RBCs), leukocyte count (WBCs), hemoglobin concentration (Hb), and Packed cell volume (PCV) were estimated using an automatic cell counter system XT (2000 IX) (Sysmex Corporation of America, Long Grove, Illinois, USA) (Jain and Zinkl, 2000).

2.3.5. Parasitological examination

Thin blood films (no.7) were prepared for the identification of hemoparasites (Salakij et al., 2012).

2.3.6. Histopathological analysis

Aseptic specimens from the liver, thymus, bursa, muscles, skin, heart, kidney, brain, and lungs were preserved in 10% neutral buffered formalin. Tissue sections of 4μm thickness were cut out of the paraffin-embedded blocks, stained with

Table 2: Oligonucleotide primers used in this study.

Target gene*	Primers sequences 5'- 3'	Annealing Temperature	Reference
Influenza A virus	AGA TGA GTC TTC TAA CCG AGG TCG TGC AAA AAC ATC TTC AAG TCT CTG	60°C	Spackman et al. (2002)
AIV H5	ACAAAGCTCTATCAAAACCCAAC TACCCATACCAACCATCTACCAT	57°C	Chaharaein et al. (2009)
AIV H9	ATCGGCTGTTAATGGAATGTGTT TGGGCGTCTTGAATAGGGTAA	59°C	Chaharaein et al. (2009)
NDV	ATG GGA AGC GGA TCA TCA TCA TC CAA CTT TGT CTG CTG CTG CTG TG	60°C	Fuller et al. (2009)
NDV-GVII	AGTGATGTGCTCGGACCTTC CCTGAGGAGAGGCATTTGCTA	60°C	Wise et al. (2004)
IBDV	TACCAATTCTCATCACAGTACCAA CGGAGGGCCCCT GGATAGTT	60°C	Charoenvisal (2021)
CIAV	GAG GAG ACA GCG GTA TCG GCG GAT AGT CAT AGT AGA TTG G	60°C	Chansiripornchai et al. (2012)
FAdV	ATTTTCAACACCTGGGTGGAGAGCA CACGTTGCCCTTATCTTGC	58^0c	Mase et al. (2021)
Cytb *(L. caulleryi)*	TGCCTGGATTATTTGGTGGT TGTCCATCCTGTACCACCAC	58°C	This study
Cytb *(L. sabrazesi)*	ATGGAGTGGATGGTGCTTCA AGTAATTACAGTTGCACCCCA	60°C	This study
SPP *(P. galinaceum)*	AGGTCGAGCGATGGGATATG CCTTGCAATGAGAACACCCC	60°C	This study
Cytb *(Haemoproteus spp.)*	TGACTGCATGTGTACAATTCCT TCTGATGTTTGAAGTGATGCAAA	58°C	This study

* Abbreviations: AIV: avian influenza virus, NDV: Newcastle disease virus, IBDV: infectious bursal disease virus, CIAV: chicken infectious anemia virus, FAdV: fowl adenovirus.

Table 3: Cycle threshold (Ct) values of identified pathogens.

Pathogens		Flock no 1		Flock no 2		Flock no 3	
		Positivity	Ct	Positivity	Ct	Positivity	Ct
Viruses*	NDV	+	23.19	+	30.11	+	31.65
	NDV-GV11	+	20.01	+	26.32	+	27.11
	AIV	+	22.31	-	-	-	-
	AIV H5	-	-	-	-	-	-
	AIV H9	+	23.61	-	-	-	-
	CIAV	+	22.12	-	-	+	18.37
	IBDV	+	18.52	-	-	+	24.02
	FAdV	-	-	-	-	-	-
Blood parasites	*L. caulleryi*	-	-	-	-	+	24.33
	L. sabrazesi	+	22.31	+	24.01	-	-
	Haemoproteus spp	-	-	+	22.98	-	-
	P. gallinaceum	+	24.02	-	-	-	-

* Abbreviations: AIV: avian influenza virus, NDV: Newcastle disease virus, IBDV: infectious bursal disease virus, CIAV: chicken infectious anemia virus, FAdV: fowl adenovirus.

hematoxylin and eosin stains, and subsequently analyzed using a light microscope (Bancroft and Gamble, 2007).

3. Results

3.1. Clinical and postmortem findings

The clinical signs were pale face, weight loss, and ruffled feathers. Some cases showed a febrile condition, greenish-whitish diarrhea, and a tendency to extend legs backward. Up on necropsy, various pathological lesions with a predominant hemorrhage in several organs were recorded (Table 1 and Figure 1, Figure 2, and Figure 3).

3.2. Molecular analysis and characterization of the collected samples

All pooled samples per flock were positive for NDV-GVII. The nucleic acid of *L. sabrazesi* was identified in two flocks (flocks no. 1 and 2), while, *L. caulleryi* was found only in one flock (flock no. 3). CIAV and IBDV co-infection was detected in flocks no 1 and 3. All examined flocks were negative for FAdV (Table 3).

3.3. Examination of blood smears

In this study, *L. sabrazesi*, and *L. caulleryi* as well as *Pl. gallinaceum* were detected. Rounded macrogametocytes of *L. sabrazesi* and the trophozoite of *P. gallinaceum* were detected in flock no.1, However, fusiform macrogametocytes and rounded immature microgametocytes of *L. caulleryi* were detected in flock no. 3 (Figure 4 A, B, C, and D, respectively).

3.4. Analysis of Hematological Parameters

Hematological parameters showed a significant decline in the Hb, PCV, and TLC, especially lymphocytic count (LC) in the three flocks (Table 4).

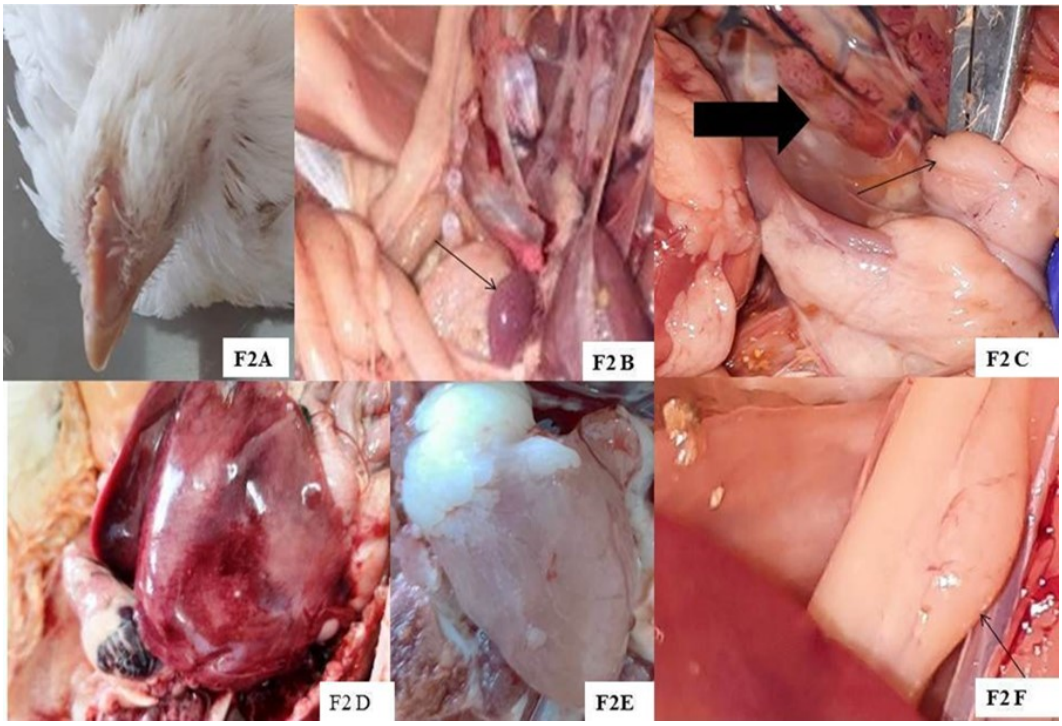

Figure 2: Post-mortem lesions of flock no 2. (A) Paleness in the comb and wattle. (B) Atrophy in the spleen. (C) Atrophy in the bursa, and paleness with hemorrhage in the kidney. (D) Subcapsular hemorrhage on the liver. (E) Paleness of the heart. (F) Enlarged cecal tonsils.

3.5. Histopathological examination

A varying degree of pathological alterations in different organs was recorded indicating the involvement of several pathogens. In, flock no.1 the lung and proventriculus had interstitially embedded megaloschizonts which are characteristic of *Leucocytozoon* (Figure 5 5 A & D). However, in flock no.2, the intestine, heart, and bursa showed interstitially lodged megaloschizonts, while other organs, including the lung, liver, spleen, heart, and skeletal muscles had severe microscopical pathological reactions toward *Leucocytozoon* (Figure 5 B & C, Figure 6 A, B, C, D, E, & F), and Figure 7 (A, B, E & I).

In flock no.3 the brain, heart, and liver expressed inflammatory reactions indicative of NDV-GVII infection. The spleen and bursa exhibited lymphocytic depletion, indicative of CIAV and IBDV (Figure 7 C, D, F, G, H & E).

4. Discussion

Co-infection in poultry farms causes serious complications in the clinical manifestation of disease due to its difficult diagnosis, There is little research on interactions between co-infecting pathogens, which further complicates the problem (Gowthaman et al., 2019). This study aimed to adopt the most confidential and reliable diagnostic methods for distinguishing between viral and avian haemosporidian involved in hemorrhagic syndrome as much as possible.

The current study identifies similarities in clinical and postmortem lesions attributable to multiple etiologies such as AIV, CIAV, IBD, FAdV, NDV, and Leucocytozoonosis. These lesions include paleness of comb and wattle, extensive hemorrhaging in various organs, skin, muscles, and legs (manifested as circular spots), variable bursal enlargement and/or atrophy, occasional thymic atrophy, pancreatic necrosis, and septicemia which align with the age of three examined broiler flocks complicating field align with diagnosis (Abou El-Azm et al., 2022; Damairia et al., 2023).

To the best of our knowledge, this study provides the first record of the detection of *L. sabrazesi* in broiler chicken flocks in Egypt. Additionally, the first reporting of co-infection DV-GVII and avian haemosporidian along with other immunosuppressive viruses is documented based on post-mortem lesions, molecular data, blood film & hematological analysis, and histopathological examination.

Currently, real-time PCR is considered a gold standard for the molecular diagnosis of different pathogens (Espy et al., 2006). As a part of this study, a new set of primers was made to target

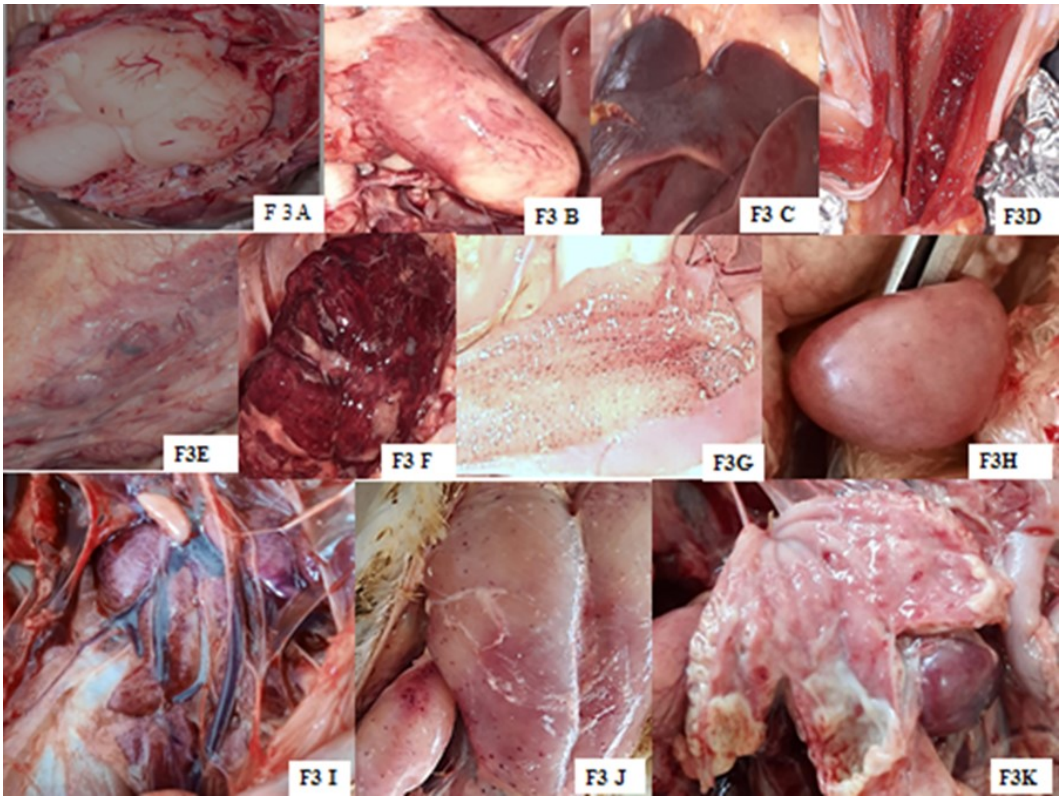

Figure 3: Post-mortem lesions of flock no 3. (A) Congestion, and hemorrhage on the brain. (B) Petechial hemorrhage, and degeneration (necrosis) on the myocardium of the heart. (C) Peripheral infarction of the liver. (D) Fragile, and congested bone marrow. (E) Petechial hemorrhage on the thymus. (F) Congested lung. (G) Hemorrhage in the rectum. (H) enlarged, and paleness in the spleen with necrosis. (I) Hemorrhage in the kidney. (J) Petechial hemorrhage on the thigh, and keel muscle. (K) Petechial hemorrhage on the papillae of the proventriculus.

four avian haemosporidians at species-specific levels (Table 2). Real-time PCR was used by several studies targeting conserved regions of rDNA of avian haemosporidian genera (Bell et al., 2015; Agbemelo-Tsomafo et al., 2023), which can screen without making a distinction between parasites genera. A *cytb* fragment is widely used to study the ecology, taxonomy, and phylogeny of avian malaria parasites (Pohuang et al., 2021).

Several studies have used *cytb* fragment primers previously. The initial set targets a comprehensive group of avian haemosporidian genera which was originally designed by Bensch et al. (2000), subsequently modified by Hellgren et al. (2004), and is extensively utilized for nested PCR. The second set was designed by Zhao et al. (2016) for *L. sabrazesi* and *L. caulleryi*. As shown in this study, we designated a new *cytb* primer set to detect and identify the most three common avian haemosporidian species infecting chickens at once by qPCR. Additionally, for the first time, we used the *SPP* gene of *P. gallinaceum* via amplification of a conserved region of the antigen delta-aminolevulinic acid dehydratase which is responsible for host erythrocyte invasion, transmission, host-specificity, and immuno-evasion of the malaria parasite (Lauron et al., 2014).

Based on molecular analysis, this study documents the first reported concurrent infection of *L. sabrazesi* and *L. caulleryi* with NDV-GVII and immunosuppressive viruses (CIAV and IBDV /AIV H9) (Table 3). Since its first detection in 2011, NDV-GVII has consistently posed a threat to the Egyptian poultry industry (Eid et al., 2022). Several researchers documented the co-infection of NDV-GVII with AIV in Egypt (Eid et al., 2022; Osman et al., 2024). Additional reports documented the co-infection of NDV-GVII with CIAV and IBDV worldwide (Sharifi et al., 2022). Concurrent infection of CIAV and IBDV was reported in Egyptian broiler flocks (Mohamed and Hammad, 2021). Co-infection by *Plasmodium spp* and *Leucocytozoon spp* demonstrated the co-existence of *Simulium spp* and mosquitoes in the rearing environment (Ola-Fadunsin et al., 2022), whereas the presence of Culicoides biting midges (*Ceratopogonidae*) facilitates *Haemoproteus spp* transmission (Valkiūnas and Atkinson, 2020).

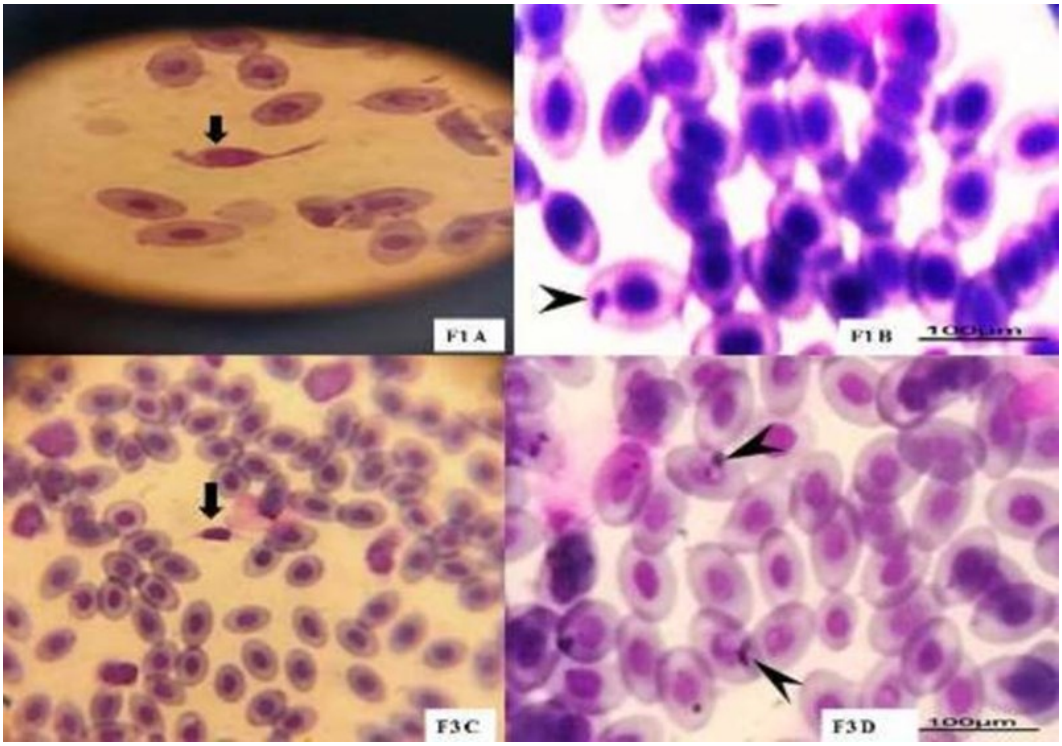

Figure 4: Blood film examination in flocks no 1 & 2. (A) Gametocyte of *Leucocytozoon Sabrazesi* in the flock no 1. (B) Trophozoites of *Plasmodium gallinaceum* in blood film of the flock no 1. (C) Gametocyte of *Leucocytozoon caulleryi* in blood film of the flock no 3. (D) Immature gametocyte of *Leucocytozoon caulleryi* in blood film of the flock no 3.

This study provided a recent descriptive interaction under field conditions and their effects on clinical, postmortem lesions, hematological analysis, and histopathological examinations correlated with cycle threshold (Ct) values which were indicative of tissue loads according to the following criteria: Ct 17- 24 highly load, 24-35 moderate load, and ≥36 no detection (Finks et al., 2023).

The majority of recorded postmortem lesions correlated with qPCR Ct values which provide an earlier tool for veterinarians to predict the most common etiological agents involved in hemorrhagic syndrome with few exceptions for AIV H9, *P. gallinaceum*, and *Haemoproteus spp.*, which exhibited negligible postmortem lesions (Table 1). Bursal lesions represented by enlarged, edematous, caseated bursa with thickened folds in flock no.1 correlated with an IBDV Ct value of 18.52 and indicated acute IBDV (Damairia et al., 2023), While in flock no.3, there were different bursal lesions including atrophy and enlargement with a CIAV Ct value of 18.3 and IBDV Ct value of 24.2 which is consistent with the findings of Damairia et al. (2023), who documented that CIAV and IBDV caused atrophy of the bursa follicles.

Leucocytozoonosis infections have been linked to circular spots of bleeding on the shank, inside the buccal cavity, thymus, peritoneal cavity, muscles, pancreas, roughly skin appearance (exhibiting a nodular morphology), and enlarged necrotizing spleen (Lee et al., 2016; Elbestawy et al., 2021; Abou El-Azm et al., 2022). The clinical and post-mortem manifestations linked to NDV-GVII included greenish diarrhea, septicemia, hemorrhage on proventriculus, rectal hemorrhage, mottled spleen, enlarged cecal tonsils, and cerebral congestion were similar to other reported findings by Abdallah et al. (2023); Osman et al. (2024), as well as atrophy of bursa and spleen (Rabiei et al., 2024).

The detected AIV H9 in flock no.1 was associated with septicemia, congested lungs, and trachea, enteritis, nephritis, and splenomegaly as reported by others (Megahed et al., 2020). The recorded CIAV clinical and postmortem lesions in this study included pallor, hemorrhage on muscles, and internal organs, and bursal atrophy, aligning with the findings of Mostafa et al. (2021); Abou El-Azm et al. (2022), congested and fragile bone marrow, along with enlarged liver and spleen, were similar to the observations of (Abdel-Mawgod et al., 2024). Furthermore, the absence of bone marrow lesions despite a high tissue titer substantiated that CIAV is directly cytotoxic for bone marrow hematopoietic corroborating the

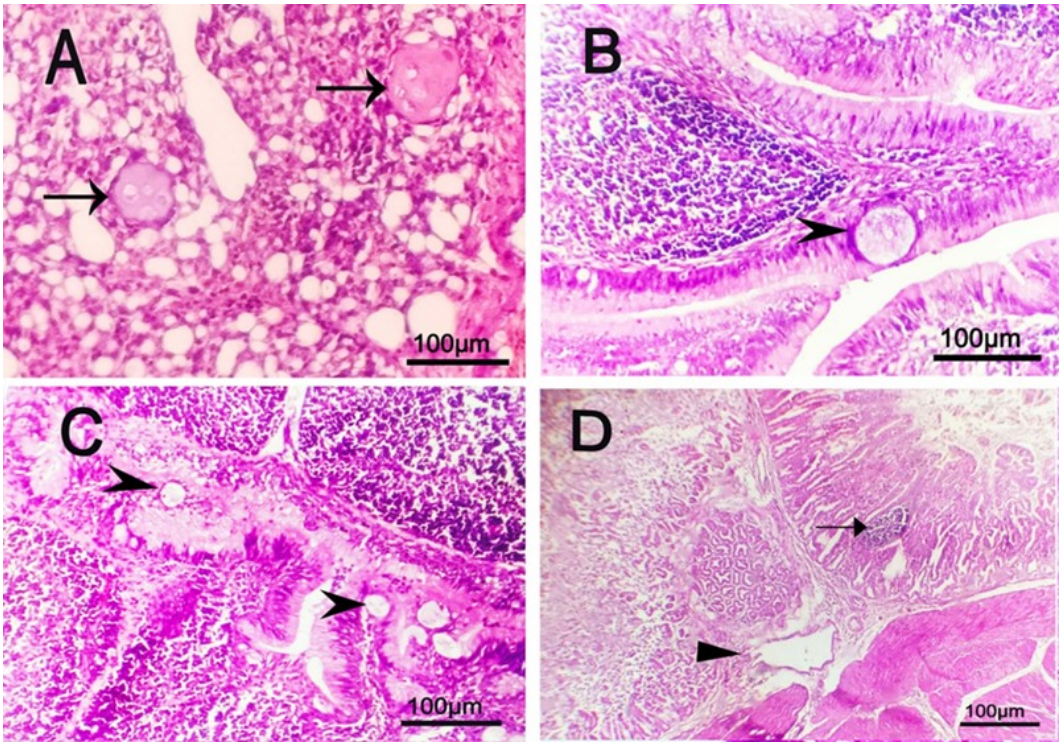

Figure 5: Photomicrograph of chicken infected with *Leucocytozoon spp* in flocks no 1 & 2 show different megaloschizont stages in different organs. A) Lung with intact interstitially embedded megaloschizonts (arrows) characterized by round, unilocular structure filled with numerous basophilic schizonts which surrounded by a capsular layer "flock no 1". (B) Bursa of Fabricius with intact embedded megaloschizont in the covering lymphoid epithelium (arrowhead) characterized by round, unilocular structure filled with numerous basophilic schizonts surrounded by a capsular layer "flock no 1". (C) Bursa of Fabricius with many intact embedded megaloschizonts(arrows) in the covering lymphoid epithelium "flock no 2". (D) Proventriculus with embedded degenerated ruptured megaloschizont (arrowhead) with irregular capsule and without schizonts. A focal area of mononuclear cell infiltration is observed (arrow) "flock no 1". Scale bar = 100 μm.

conclusions of (Haridy et al., 2012). Ultimately, these findings evidenced that CIAV accelerates susceptibility to multiple infections due to its immunosuppressive effects (lymphocyte depletion), and exacerbates their pathogenicity even during interactions with each other as noted by Erfan et al. (2019).

Hematological analysis can effectively elucidate the physiological condition of birds either under stress or disease efficiently (Malvat et al., 2020). In this study, a notable decrease in primarily blood parameters, as detailed in Table 4. CIAV-infected flocks no. 1 and 3, showed a marked reduction in hematocrit values (Mostafa et al., 2021), along with a significant decline in Total Leucocytic Count (TLC), especially lymphocytes and monocytes (Vaziry et al., 2011). Mononuclear cells are the main target cell for CIAV, which exerts a cytolytic effect on lymphocyte populations resulting in atrophy and depletion of lymphoid organs (Umar et al., 2017). The marked decline in Hb, and TLC, was accompanied by different hypotheses: intravascular hemolysis (Eze et al., 2014), releasing merozoites from megaloschizonts (Goto et al., 1966), in addition to the direct effect of CIAV on a hematopoietic progenitor cells of the bone marrow (Hoop and Reece, 1991). Slight elevation of heterophils with a marked decline in TLC in the flock no. 2, despite the presence of two blood protozoa and NDV G-VII, which aligns with the findings of Campos et al. (2014) regarding the initial phase of infection, along with lymphoid depletion caused by NDV-GVII (Rabiei et al., 2024). Blood parasites do have not a significant impact on avian eosinophils in contrast to mammals (Campbell and Ellis, 2013).

Thin blood smears are an economical and dependable technique for diagnosing blood parasites but lack sensitivity (Valkiunas et al., 2008). We interpreted our results in alignment with the conclusions of (Zhao et al., 2016; Nguyen, 2019). The recorded low number of gametocytes of *L. sabrazesi*, and *Plasmodium* in flock no. 1, in addition to *L. caulleryi* in flock no.3, and the absence of both *Haemoproteus spp* and *L. sabrazesi*

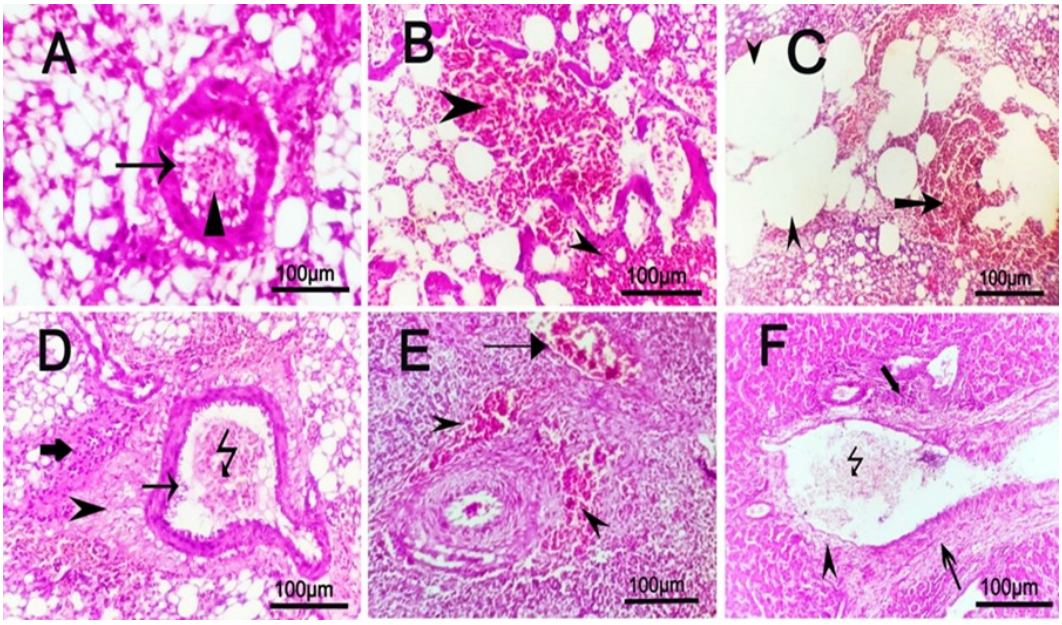

Figure 6: Photomicrograph of different lesions in different organs in flock no 2 infected chickens. (A) lung with vascular congestion (arrowhead), and vascular endotheliosis (arrow). B) lung with massive interalveolar extravasated erythrocytes (focal hemorrhage) (arrows head). C) lung with broken alveoli (emphysematous reaction) (arrows head) and interalveolar extravasated erythrocytes (arrow). D) Massive destruction of the secondary lamellae accompanied by clubbing of the few remaining ones (black lung with massive perivascular fibrosis (arrowhead) and inflammatory cells infiltration predominantly eosinophile (thick arrow), and vascular congestion (zigzag arrow) with endotheliosis (arrow). E) spleen with perivascular congestion (arrow), and hemorrhage (arrows head). F) liver with perivascular vacuolation of hepatocytes (arrowhead), fibrosis (thin arrow) with inflammatory cells infiltration predominantly lymphocytes (thick arrow), and vascular congestion (zigzag arrow). Scale bar = 100 μm.

in flock no. 2, despite the low Ct value may be attributed to two potential explanations; the incomplete life cycle of the parasites within the host (Valkiūnas and Iezhova, 2023), or the inadequate number of RBCS for gametocyte development (Pattaradilokrat et al., 2014).

This study documented various lesions induced by *Leucocytozoon spp* through histopathological examination, revealing numerous megaloschizonts in organs (Figure 5 & Figure 6), such as the lung, proventriculus, bursa, intestine, and heart (Lee et al., 2016; Pohuang et al., 2021). Additionally, vascular congestion and thrombosis were detected in flock no. 2 affecting the lungs, liver, spleen, heart, and skeletal muscles, meanwhile in flock no.3 it involved the brain, liver, and spleen (Figure 6 & Figure 7) (Elshahawy et al., 2024). Furthermore, *Leucocytozoon spp* causes colonization, blockage, and thrombosis resulting in multiple focal areas of necrosis and ischemia, ultimately resulting in cardio-respiratory failure and mortality (Donovan et al., 2008). The absence of megaloschizonts of *Leucocytozoon spp* in flock no.3 may be attributed to chronic parasitemia which is marked by challenges in locating tissue-persistent meronts, analogous to the previous findings by de Francisco et al. (2023).

Notable histopathological lesions of NDV-GVII were detected in multiple organs including the brain, liver, and heart in flock no. 3, (Figure 6), and lungs in flock no. 2, which exhibited congestion, perivascular edema, necrosis, fibrosis, and ruptured alveoli, similar (Al-Murshedy et al., 2023).

The detected lymphoid depletion particularly in the bursa and spleen of flocks no. 2, and 3, are consistent with the findings reported by Rabiei et al. (2024) for NDV G-VII, Singh et al. (2015) for IBDV, and Hosokawa et al. (2020) for CIAV. Therefore, based on histopathological changes, these organs hold minimal significance in the context of natural field co-infection.

In this context, we accommodate a preliminary, straightforward, and dependable strategy for detecting and characterizing several etiological agents involved in hemorrhagic syndrome based on the correlation between the different diagnostic techniques.

Avian haemosporidian is properly diagnosed using qPCR (Lee et al., 2016) and histopathology while hematology particularly WBCs and blood film, exhibited low significance in this study (Bell

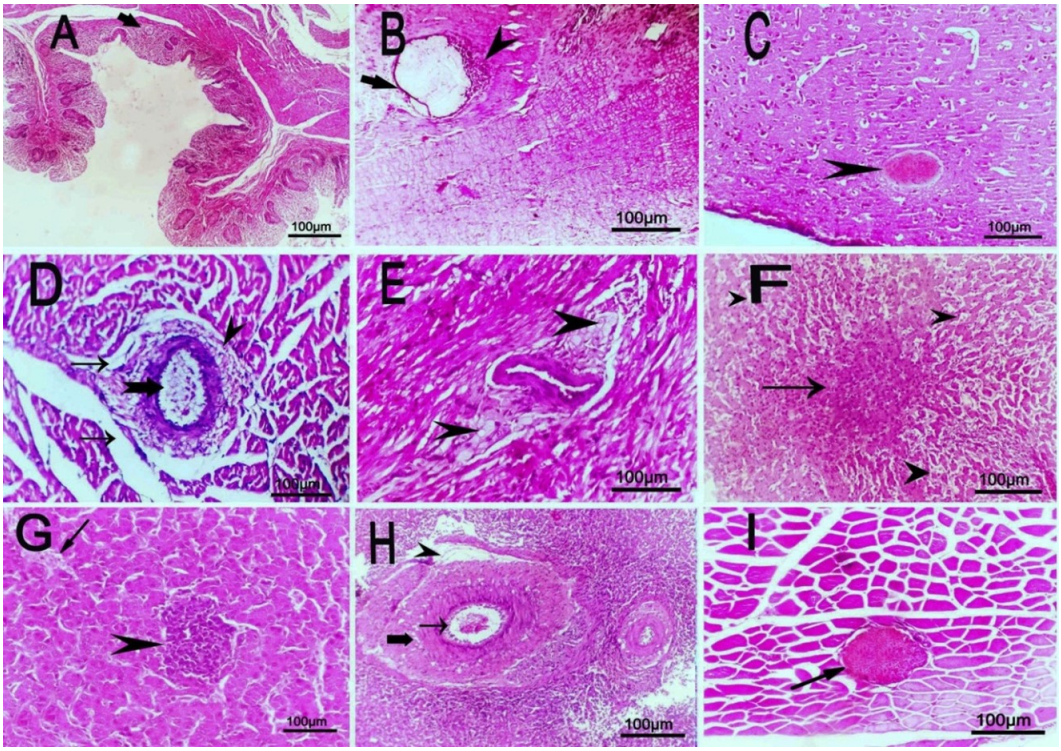

Figure 7: Photomicrograph of different lesions in different organs in flocks no 2& 3 infected chickens. (A) Intestine with submucosal embedded megaloschizont (arrow) as round, and unilocular structures filled with numerous basophilic schizonts surrounded by a capsular layer "flock no 2". (B) Heart with embedded early degenerated megaloschizont (arrow) with an irregular wall filled with numerous basophilic schizonts and disorganization of cardiac muscle fiber "flock no 2". (C) Brain with congestion of cerebral blood vessels (arrowhead) "flock no 3". D) Heart with perivascular edema (arrows) and fibrosis (arrowhead) and congestion (thick arrow) "flock no 3". (E) Heart with perivascular vacuolation (arrows head) of cardiac muscle fibers (arrowhead) "flock no 2". (F) Liver with a focal area of hepatic necrosis (arrow) with dilated congested sinusoids and atrophied hepatocytes (arrowhead) "flock no 2". (G) Liver with a focal area of inflammatory cell infiltration predominantly lymphocytes (arrow) "flock no 3". H) Spleen with vacuolation of vascular tunica intima (thick arrow), endotheliosis (thin arrow), and perivascular edema (arrowhead) "flock no 3". (I) Skeletal muscle with severe congestion of blood vessels (arrow) "flock no 3".Scale bar = 100 mum.

et al., 2015; Zhao et al., 2016; Seidl et al., 2024). The efficacy of qPCR in detecting low-intensity blood film infection and histopathology are crucial for diagnosing leucocytozoon in chickens. The deficiency of sensitivity and specificity of blood film was noted by Doctor et al. (2016). Our findings revealed a notable correlation between the post-mortem lesions, blood smear, and histopathological analysis. Therefore, primary determination of the best time for accurate blood film expression can be established depending on the presence of extensive hemorrhage, necrosis of the heart and spleen with their paleness macroscopically, and the existence and early destruction of the megaloschizont at the microscopic level, as well as, the severe inflammation following its release (chronic stage) (Suprihati et al., 2020). However, the minimal impact on WBCs was reported by Wiegmann et al. (2021).

This study confirmed the successful diagnosis of CIAV based on hematological analysis particularly examining PCV, lymphocytes, and monocytes (Mostafa et al., 2021) and q PCR (Al-Azawy et al., 2020). The proper diagnosis of IBDV primarily relies on macroscopic lesions in the bursa (Damairia et al., 2023), followed by qPCR for subsequent confirmation (Adel et al., 2024). Even though CIAV leads to bursal atrophy but does not replicate within the bursa (Dey et al., 2019). Eventually, microscopic inspection proved ineffective as a distinguishing tool, as previously in cases of mixed infection by CIAV and NDV-GVII as previously mentioned.

5. Conclusion

This study reports the co-infections of NDV GVII and *Leucocytozoon spp* (*L. sabrazesi* and *L. caulleryi*), alongside other viruses such as AIV H9, IBDV, CIAV, and blood protozoa (*Haemoproteus spp.*, *Plasmodium spp.*), occurring as triple infections or more in Sharqia Province. Moreover,

Table 4: Blood parameters of investigated flocks.

Paramaters[a]	Flock1		Flock 2		Flock 3		Normal ranges	
RBCs counts &indices								
RBCS	2.7×10^6 - 5.2×10^6		3.12×10^6		2.23×10^6 - 3.7×10^6		2.50-3.50x 1012/μl[b]	
Hb	5.6 -5.9		10.80		9.1-10.2		11.20 – 17.10 g/dL[b]	
PCV	20.3-21.3		39.10		23.6-27.1		35.00-55.00 %[b]	
MCV	122.4-124.1		133.40		58.9-107.0		104.0-135.00 fl[b]	
MCH	33.3-33.7		36.80		21.1-36.6		33.00- 47.0 pg[b]	
MCHC	27.20		28.60		23.3- 35.3		30.20-36.20 g/dl[b]	
RDW	17.1-19.8		17.50		12.1-16.2		-	
Platelet count								
Manual/μl	18×10^3-20×10^3		375×10^3		5×103 - 10 $\times10^3$		36.78 $\times10^3/\mu$l[c]	
Total leucocytic count								
WBCS/μl	5.9×10^3 - 6.5×10^3		71.8×10^3		8.3×10^3 -14.3×10^3		80.33 $\times10^3/\mu$l[c]	
Differential leucocytic count								
(%,$\times10^3/\mu$l)	$\times10^3$	%	$\times10^3$	%	$\times10^3$	%	$\times10^3/\mu$l	%
Heterophils	42-43	2.48- 2.80	68.00	48.82	28- 77	2.33-11.02	15.0 - 50.0	-
Band(staff)	3-4	0.18- 0.26	3.00	2.15	3-5	0.25-0.72	-	-
Segmented	39.00	2.30-2.54	65.00	46.67	25-72	2.08- 10.30	-	-
Lymphocytes	50-55	3.25	23.00	16.51	18-63	2.49- 5.23	45.0 - 70.0[b]	70. 83[c]
Monocytes	2-5	0.12- 0.33	6.00	4.31	4-7	0.52-0.58	5.0 - 10.0[b]	3.21-5.13[c]
Eosinophils	1-2	0.06-0.13	2.00	1.44	1-2	0.09-0.17	1.5 - 6.0[b]	-
Basophils	0.00	0.00	1.00	0.72	1.00	0.09	Rare[b]	-

[a] Abbreviations: RBCS: red blood cell, Hb: hemoglobin, PCV: packed cell volume /hematocrit, MCV: mean cell volume, MCH: mean cell hemoglobin, MCHC: mean cell hemoglobin concentration, RDW: RBCS distribution width, WBCS: white blood cells, DLC: differential leucocytic count.
[b] Reference values according to Jain (1993).
[c] Reference values according to Muneer et al. (2021).

this is the first documentation of *L. sabrazesi* in broiler chickens in Egypt.

Despite routine vaccination, contagious and immunosuppressive viruses, such as NDV-GVII, AIV H9, CIAV, and IBDV persist, along with the co-existence of *Leucocytozoons*, *P. gallinaceums*, and *Haemoproteuss spp.*, which indicates that vectors are abundant during the warmer seasons. Future recommendations include conducting surveillance and epidemiological studies on existing co-infected agents to continuously evaluate the effectiveness of preventative and control hygiene measures and their enhancements to mitigate and eliminate such pathogens.

Article Information

Ethical Approval. This study was approved by the Institutional Animal Care and Use Committee of Zagazig University (ZU-IACUC committee) in Egypt's guidelines collection of samples from nonliving animals (approval No. ZU-IACUC/2/F/39/2024).

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Conflict of Interest. The authors declare no conflict of interest.

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