# PATHOLOGICAL AND MOLECULAR INVESTIGATION OF HEPATOMEGALY AND SPLENOMEGALY SYNDROME IN POULTRY IN CHATTOGRAM

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**Khulshi-4225, Chattogram, Bangladesh**

**JUNE, 2023**

***Dedication***

*To my Parents*

*who always valued education*

*above everything else*

## Authorization

I hereby declare that I am the sole author of the thesis submitted in fulfillment of the requirements for the Degree of Master of Science (MS) in the Department of Pathology and Parasitology (DPP), Faculty of Veterinary Medicine, Chattogram Veterinary and Animal Sciences University (CVASU). I authorize CVASU to lend this thesis or to reproduce the thesis by Xeroxing or any other means, at the request of any other institutions or individuals for the purpose of scholarly research.

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**This is to certify that we have examined this thesis and have found that it is complete and satisfactory in all respects, and that all revisions required by the thesis examination committee have been made.**

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## List of abbreviations and symbols used.

| Abbreviation & symbol | Elaboration |
|---|---|
| $^0C$ | Degree Celsius |
| DLS | Department of Livestock Services |
| gm | Gram |
| mg | Milligram |
| min | Minute (s) |
| ml | Milliliter |
| ALV | Avian Leukosis Virus |
| MDV | Marek's Disease Virus |
| PCR | Polymerase Chain Reaction |
| % | Percentage |
| EMB | Eosin Methylene Blue |
| sec | Second (s) |
| XLD | Xylose Lysine Deoxycholate |
| µg | Microgram |

# Abstract

Enlarged liver and spleen are notable pathological lesions in poultry indicative of many infectious diseases that leads to economic losses due to high mortality rates and reduced egg production. Various bacteria, viruses, and infectious agents are associated with these notable lesions in the poultry. In this study, we conducted a cross-sectional study using enlarged liver and spleen samples from 35 different dead chickens (15 layers and 20 broilers). These samples were collected after postmortem examination from the dead birds brought to the department of Pathology and Parasitology at Chattogram Veterinary and Animal Sciences University (CVASU), by different poultry farm owners in Chattogram district for diagnosis and treatment purposes. Among those samples 4 different layer birds had whitish nodular lesions in the liver and spleen and tentatively diagnosed as Marek's disease (MD) or Lymphoid leukosis (LL). However, in other cases congestive lesions were markedly observed in liver and spleen and were diagnosed as *E. coli,* Salmonella or other viral infections. There were no perihepatitis or hemorrhage in abdominal cavity in any cases. After necropsy, swab samples from liver and spleen were initially inoculated into selenite cystine broth and blood agar, and subsequently streaked onto EMB and XLD agar for the isolation and identification of bacterial colony. A part of the samples were also preserved in 10% buffered formalin for histopathological examinations, and in -20$^0$C for molecular work. After bacteriological culture 17 (48.5%) samples showed predominantly positive results for *E.coli* on EMB agar, while no *Salmonella sp* were positive on XLD agar, based on colony characteristics. For molecular confirmation DNA was extracted from those 4-layer bird samples (liver and spleen) that were suspected MD/LL, and PCR was done initially using ICP4 gene primers for MD virus. Finally, it's found that 3 samples were amplified for ICP4 gene and confirmed to be Marek's disease. But due to limitations RNA couldn't be extracted and RT-PCR couldn't be done to check another sample with nodular lesion which might be LL or due to other RNA viruses. Furthermore, the liver and spleen samples with nodular lesions and some other samples were investigated for histopathological lesions which reveals notable findings include- severe congestion and hemorrhages, neoplastic foci with the accumulation of pleomorphic cells specially lymphoblast, various sized lymphocyte, and macrophages in the liver. In spleen, various sized lymphocytes along with extended white pulp also found which is composed of reticular cells indicative

for extensive inflammatory reaction. In conclusion, molecular diagnosis is necessary for hepatomegaly and splenomegaly along with the histopathological examinations that is associated with any lymphoproliferative lesions.

***Keywords:*** *hepatomegaly, splenomegaly, nodular lesion, Mareks disease, PCR, histopathology, chickens*

# CHAPTER I

# INTRODUCTION

The growth of the poultry sector in Bangladesh faces significant challenges, with diseases being recognized as a primary factor responsible for a 30% annual chicken mortality rate (Das et al., 2005). The prevalence of these diseases varies depending on geographic and climatic conditions, seasons, poultry breeds, and the age of the birds. In some instances, a particular disease may have a negligible impact on an area, while at other times, it can wreak havoc (Rahman et al., 2004). Bangladesh, being a tropical country, is particularly susceptible to diseases such as colibacillosis, salmonellosis, Newcastle disease (ND), infectious bursal disease (IBD), and coccidiosis, which are more prevalent in its poultry population. These diseases are highly contagious and tend to affect young chickens, often leading to high mortality rates (Lukert and Saif, 1997). Notably, bacterial diseases like pullorum disease (PD), fowl cholera (FC), and colibacillosis (Samad, 2000) contribute significantly to morbidity and mortality in poultry industry of Bangladesh.

In addition to bacterial diseases, viral infectious diseases, including avian influenza (AI), Newcastle disease (ND), infectious bursal disease (IBD) (commonly known as Gumboro disease), and Marek's disease, have been identified as major hindrances to the growth of the poultry sector in Bangladesh (Mshelia et al., 2016). These diseases impose substantial economic losses on farmers (Wakawa et al., 2014)

However, In the poultry industry, several diseases have a significant impact on the liver and spleen, leading to their enlargement and subsequent malfunction, often resulting in bird mortality (Abraham-Oyiguh et al., 2014). Among viral diseases, both broiler and layer chickens can experience hepatomegaly (enlarged liver) primarily due to fowl adenovirus and hepatitis E virus. Additionally, lymphoid leukosis virus and Marek's disease virus can lead to hepatomegaly and splenomegaly (enlarged spleen) specifically in layer chickens. Moreover, bacterial diseases, such as salmonellosis and colibacillosis, also contribute to hepatomegaly in both layer and broiler birds (Balami et al., 2015; Calnek, 2001)

Chickens affected by hepatomegaly and splenomegaly typically exhibit symptoms like enlarged liver and spleen, along with the presence of serosanguineous fluid in their coelomic cavities. This health issue often results in reduced egg production and elevated mortality rates (Meng and Shivaprasad, 2013). Histopathological examinations may reveal characteristic changes such as extensive coagulative necrosis, vasculitis, hemorrhage, amyloid deposition, and non-specific hepatitis (Agunos et al., 2006). Infectious diseases of viral and bacterial origin that affect the liver and spleen can impose significant economic losses on poultry farmers due to increased mortality and decreased egg production (Morrow et al., 2008). Therefore, it is essential to research the diseases and their causative agents that lead to hepatomegaly and splenomegaly for better understanding and management of these health challenges.

However, there is a limited body of research available that investigates the effects of various microorganisms on poultry resulting in hepatomegaly and splenomegaly. Consequently, this research was undertaken to identify the causal agents along with pathological correlation in hepatomegaly and splenomegaly lesions in commercial broiler and layer chickens in Bangladesh.

# CHAPTER II

# REVIEW OF LITERATURE

## 2.1 Hepatomegaly and splenomegaly syndrome

Hepatomegaly and splenomegaly syndrome is a disease diagnosed in both broiler breeder hens and commercial egg-laying hens. Big liver and spleen disease (BLS) is an infectious, transmissible disease. The etiology of this syndrome is not known but, because of the vasculitis and the deposition of amyloid, immune-mediated mechanisms have been hypothesized (Barnes, 1997). Big liver and spleen disease (BLS), recognized in Australia since 1980 and is considered the most economically significant disease affecting commercial broiler breeder flocks in Australia (Handlinger and Williams, 1988). The syndrome is considered economically important to the major integrated companies of broiler breeders in that country. It is characterized by increased mortality, decreased egg production, and lesions in the liver and spleen. Affected birds have hepatomegaly and splenomegaly, with marked inflammation, vasculitis, hemorrhage, and deposition of amyloid in both organs (Shivaprasad, 2003). The condition has been recognized throughout North America. In Canada, it was first reported in 1991 (Ritchie and Riddell, 1991), but it disappeared after 1994.

## 2.2 History of Hepatitis and splenomegaly syndrome

Hepatitis and splenomegaly syndrome is a disease diagnosed in both broiler breeder hens and commercial egg-laying hens. Big liver and spleen disease (BLS) is an infectious, transmissible disease. Big liver and spleen disease (BLS), recognized in Australia since 1980 and is considered the most economically significant disease affecting commercial broiler breeder flocks in Australia (Handlinger and Williams, 1988). The syndrome is considered economically important to the major integrated companies of broiler breeders in that country. It is characterized by increased mortality, decreased egg production, and lesions in the liver and spleen. Affected birds have hepatomegaly and splenomegaly, with marked inflammation, vasculitis, hemorrhage, and deposition of amyloid in both organs (Shivaprasad, 2003). The condition has been recognized throughout North America. In Canada, it was first reported in 1991 (Ritchie and Riddell, 1991), but it disappeared after 1994. In Bangladesh, such kind of disease

is not described yet. Many diseases in poultry cause hepatomegaly and splenomegaly in BD.

### 2.3 The etiology of hepatomegaly and splenomegaly syndrome

The etiology of this syndrome is not known but, because of the vasculitis and the deposition of amyloid, immune-mediated mechanisms have been hypothesized (Tablante et al., 1994). The disease is infectious, and the organism involved is presumed to be a virus (Handlinger and Williams, 1989). Many viruses and bacteria result in hepatomegaly and splenomegaly in both layer and broiler. FAdVs cause a variety of diseases in chickens, and inclusion body hepatitis (IBH), hydropericardium hepatitis syndrome (HHS), and gizzard erosion and ulceration are the most important of them. Avian leukosis viruses (ALVs) cause neoplasms in chickens, commonly known as lymphoid leukosis (big liver disease, lymphatic leukosis, visceral lymphoma, lymphomatosis, and visceral lymphomatosis) (Payne and Fadly, 1997). Marek's disease (MD) caused by cell-associated MD herpes virus (MDV), a member of the subfamily Alpha Herpesviridae of the family Herpesviridae (Calnek and Witter, 1997) is characterized by several conditions such as lymphomas of visceral organs (ovaries, liver, spleen, and kidneys). Among poultry diseases, avian colibacillosis caused by Escherichia coli (E. coli) is considered one of the principal causes of morbidity and mortality either as a primary or as a secondary pathogen (Kabir, 2010). Detailed postmortem examinations revealed diffuse splenomegaly and hepatomegaly with multifocal greyish areas on their surfaces while the diffusely enlarged kidneys were congested with mottled pale appearance (Abalaka et al., 2017). Salmonella belongs to the Enterobacteriaceae family with more than 2300 serovars and causing Salmonellosis. Avian salmonella infection occurs in poultry either in acute or chronic form by one or more members of the genus Salmonella, under the family Enterobacteriaceae (Hofstad et al., 1984). At necropsy, the livers were invariably enlarged, soft in consistency, congested, and showed bronze discoloration, and it was found that infected with Salmonella spp. The gross lesions were similar to the findings of Shivaprasad (2000).

#### 2.3.1 Mareks Disease virus (MDV)

Marek’s disease virus serotype 1 (MDV-1) is the causative agent of Marek’s disease (MD), a neoplastic disease in poultry that results in the formation of lymphomatous

lesions in nerves and visceral organs (Baigent and Davison, 2004). MDV-1 (also called Gallid herpesvirus 2) is a member of the Herpesviridae family, subfamily Alphaherpes virinae, and genus Mardivirus (Davison and Nair, 2004; Biarnes et al., 2013). Primary infection in birds occurs via inhalation of virus particles into the respiratory tract. Viral replication in the lungs stimulates immune cell infiltration whereby MDV preferentially infects adaptive immune system cells. Secondary infection and semi-productive viral replication result in an initial acute cytolytic phase in lymphocytes resulting in immunosuppression (Calnek, 2001; Nair, 2005). The virus becomes latent at 6 to 7 days of post-infection, allowing for immune evasion; the MDV genome integrates into the genome of CD4+ T lymphocytes without detectable expression levels of the potential antigenic proteins, allowing for systemic dissemination to organs, peripheral nerves, and feather follicles (Baigent and Davison, 2004). Fully productive viral replication occurs only in feather follicle epithelium which, when sloughed off and disseminated with air currents, becomes the primary source of infectious viral particles in susceptible birds.

#### 2.3.2 Avian leukosis viruses (ALVs)

Avian leukosis viruses (ALVs) in chickens belong to the leukosis/sarcoma (L/S) group of avian retroviruses, and they are categorized into six subgroups: A, B, C, D, E, and J (Payne and Fadly, 1997). These viruses can induce various neoplastic conditions. In natural settings, lymphoid leukosis (LL) is the most frequently occurring neoplasm caused by ALVs. LL typically originates in the bursa of Fabricius and can metastasize to other visceral organs, where malignant lymphocytes proliferate to form tumors (Payne & Fadly, 1997). Among the ALV subgroups, subgroup A is the most commonly isolated from affected flocks (Payne & Fadly, 1997). LL generally manifests between the 14th and 30th weeks of age in affected chickens, with the highest incidence occurring around sexual maturity (Vegad et al., 2001). However, instances of avian leukosis outbreaks have also been reported in younger chickens (Payne LN et al., 2000). Transmission of ALV can occur both horizontally and vertically (Payne et al., 2000).

### 2.3.3 Bacteria (*Escherichia coli*)

*E. coli* is a gram-negative, non-acid-fast, uniform staining, non-spore-forming bacillus that grows both aerobically and anaerobically and may be variable in size and shape.

Many strains are motile and have peritrichous flagella. *E. coli* is considered a member of the normal microflora of the poultry intestine, but certain strains, such as those designated as avian pathogenic *E. coli* (APEC), spread into various internal organs and cause colibacillosis characterized by systemic fatal disease (Luna, 1968). The organisms of E. coli are divided into pathogenic and nonpathogenic based on their ability to cause diseases. Pathogenically, *E. coli* strains are due to the presence of one or more virulence factors including invasiveness factors invasions, heat labile, heat-stable enterotoxins, neurotoxins, and colonization factors or adhesins. Pathogenic E. coli is divided into two types namely enteropathogenic *E. coli* and uropathogenic E. coli. Further pathogenic E. coli is grouped into enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli (EAggEC*), enterohaemorrhagic E. coli (EHEC).

*E. coli* is considered a member of the normal microflora of the poultry intestine, but certain strains, such as those designated as APEC, spread into various internal organs and cause colibacillosis characterized by systemic fatal disease (De Carli et al., 2015). It is a major cause of respiratory and septicemic diseases in broiler chickens causing mortality of less than 5% and morbidity over 50% but in layer, it affects the reproductive tract failing egg productivity and fertility (Barens and Gross, 1997). It may cause about 28% death in Sonali variety birds of Bangladesh (Biswas et al., 2006). E. coli infections cause many clinical manifestations such as air saculitis, pericarditis, septicemia, and death of the birds (Hofstad et al., 1984). The infection has also been extended to various parts and organs such as skin, joints, eyes, head, blood, heart, yolk sac, peritoneum, etc. (Barens et al., 1997).

### 2.3.4 Bacteria (*Salmonella sp*)

Avian salmonella infection occurs in poultry either in acute or chronic form by one or more members of the genus Salmonella, under the family Enterobacteriaceae (Hofstad et al., 1984). There are mainly two types of non-motile avian Salmonella spp. Namely, *Salmonella gallinarum* and *Salmonella pullorum*, are responsible for fowl typhoid (FT) and pullorum disease (PD) in poultry respectively. *S. gallinarum* and *S. pullorum* are non-flagellated, non-spore-forming, non-capsulated, gram-negative plump rods (Shivaprasad, 2000; Rajagopal and Mini, 2013) capable of producing septicemic disease in most domestic and wild birds all over the world. Mortality in chickens has

been reported as 0 to 100% by PD and 10 to 93% by FT. Increased mortality, anorexia, sudden drop in egg production, and white or yellow diarrhea are the characteristic clinical signs of the diseases.

### 2.4 Pathological features of hepatomegaly and splenomegaly syndrome

Many of the chickens exhibited feather loss, akin to premature molting, which persisted for several weeks even after recovering from the illness. Upon post-mortem examination, all the deceased birds appeared to be in good health. The most commonly observed pathological finding was an enlarged spleen, accompanied by an enlarged and fragile liver. Other noted lesions included bluish discoloration of the head, the presence of fluid in the pericardial sac surrounding the heart (hydropericardium), congestion and fluid accumulation in the lungs (pulmonary congestion and edema), inflammation of the intestine (enteritis), regression and congestion of the ovaries, and swollen kidneys. Microscopically, the following tissue abnormalities were identified: congestion and fluid accumulation in the liver, along with multiple areas of tissue necrosis and an increase in lymphoid tissue around blood vessels; widespread tissue necrosis in the spleen, with a notable increase in macrophage activity; and congestion and lymphoid tissue infiltration within the kidney interstitium. These findings, as reported by Paola-Massi et al. (2016).

In the case of lymphoid leukosis, chickens were manifested with the symptoms of enlarged liver and spleen, decreased egg production, together with lymphocyte infiltration in the portal vein peri phlebitis. Meanwhile, we found edema, congestion, and homogenous eosinophilic material in the liver cells (Moru et al., 2022). Anatomic analysis showed that most of chickens had enlarged livers, twice to three times bigger than normal ones. The livers were soft, mottled, and fragile, containing many miliary nodules, and hemorrhagic spots, stippled with red, yellow foci on the surface. The spleen was also mottled and enlarged, from mildly to severely (Moru et al., 2022)

Histopathological examinations of chicken liver tissue revealed the presence of focal lymphocytic infiltration, including heterophils and mononuclear inflammatory cells. Other observed features included cell edema, congestion, and the accumulation of homogeneous eosinophilic material, as well as the presence of amyloid in the interstitial spaces of certain liver cells. Notably, no necrotic changes were observed in hepatocytes. In the kidneys, signs of renal congestion and renal tubular dilatation were

evident, indicating alterations in kidney function. Meanwhile, the spleen displayed characteristics of atrophy, accompanied by a reduction in the number of lymphocytes. These histopathological findings, as documented by Moru  et al. (2022).

In the case of Marek's disease infections, gross lesions in various organs include variably sized grayish-white to yellow obvious tumor-like nodular lesions in various visceral organs such as the Liver, Spleen, Kidney, Heart, Proventriculus, Ovary, Lungs, Trachea, and nodular lesions in the skin which were suggestive of Marek's disease infections (Gopal et al., 2012). The liver was enlarged, and friable with diffusely distributing variable-sized discrete grayish-white nodules, a few nodules coalesced with each other forming big nodules (Panda et al., 1983).

The cut surface revealed the involvement of parenchyma. The spleen was enlarged to 3-4 times its normal size with a diffuse white or grayish discoloration. Ovaries were grayish-white in color with multiple nodular growths and marked enlargement with a cauliflower-like appearance (Kobayashi et al., 1986). Kidneys were enlarged, pale, and had pinpoint whitish nodules. The proventriculus was thickened with tumorous growth. In some cases, the lungs and trachea were also affected with a similar type of enlargement. The tumors were soft, smooth, grayish, and creamy white in color with or without areas of necrosis. The cutaneous form was characterized by nodular lesions at the base of feather follicles (Balachandran et al., 2009).

In most liver sections marked degree of enlargement of vessels with the presence of tumor emboli indicated metastasis to various organs and malignancy. In a few sections, in addition to neoplastic changes, infiltration of heterophils, variable degrees of degenerative changes, necrosis, and edema (Swathi et al., 2012). In spleen sections, a clear demarcation between the normal lymphocytes and neoplastic cells was lost and were found mostly perivascular, and much thickening of the blood vessels was noted. (Arulmozhi et al., 2011)

In colibacillosis, the most obvious clinical signs were diarrhea, depression, soiling of cloaca with semisolid cheesy material, respiratory distress (coughing, sneezing), reduced egg production, loss of condition, and death. Similar types of findings were described by Calnek et al. (1997), Chauhan (2003), and Vegad and Katiyar (2003). Recorded postmortem lesions were omphalitis and fluid accumulation in the peritoneal cavity of chicks, dark-colored swollen liver and spleen, pericarditis, perihepatitis,

hemorrhagic enteritis with fluid accumulation in ligated intestinal loops and diarrhea, arthritis, panophthalmitis, and salpingitis in some case (Nakamura et al., 1985).

Pathological observations in the present study in which E. coli organisms were isolated revealed thickening of the pericardium, epicardium, and hepatic area just above the surface of the liver due to the accumulation of fibrinous exudate. These lesions were consistently present in 4-week-old old birds. In the liver, the fibrinous exudate was in the form of layer/film which in 95% of cases was separated easily from the hepatic surface i.e. hepatic capsule. If n 27% of cases, fibrinous exudate was also the event in the abdominal cavity particularly on the serosal surface of the gastrointestinal tract including gizzard and mesentery. Air sacs were cloudy with deposition of fibrinous mass particularly in the birds more than 3 weeks, of age. The heart, liver, lungs, spleen, and kidneys were found congested in 95% of cases.

Histopathological changes in the liver and heart were of subacute type. There was a large amount of fibrinous exudate on the surface of the f liver consisting of heterophils and lymphocytes. It appeared that the hepatic capsule was affected. However, inflammatory cells, fibrin and degenerative changes in hepatocytes were the evident in liver parenchyma. Fibrinous pericarditis was the most common lesion, though it varied in degree in different age groups. Accumulation of severe fibrinous exudate was noticed in pericardiam. So, involvement of epicardium could not be excluded. These rethe sults ithe ndicate that in heart both pericardium and epicardium were affected whereas in the liver only hepatic capsule was affected. These differences might be due to the histological differences between hepatic capsule and hepaticum (Eurell and Fappier., 2007). Hepatic capsule is composed of fibrous connective tissue with poor cellular elements and blood capillaries Therefore, the adhesions between hepatic peritoneal sac and hepatic capsule might not be so strong and thus fibrinous layer accumulated on the surface of liver could easily be removed but not so in the case of the heart. Fibrinous inflammation was observed in the liver and heart due to colibacillosis.

The most common clinical signs of Salmonellosis were drowsiness, huddled together, poor growth, chalky white diarrhea with pasted vent, dehydration, reduced egg production, and death (Calnek et al., 1997). After necropsy, the gross lesions were observed as peritonitis, unabsorbed yolk, discrete, small, white, necrotic foci in the

liver which became swollen and fragile with distinctive coppery bronze sheen on the surface, turbid yellow color fluids in the peritoneal cavity and irregular, hemorrhagic ova with prominent thicken stalks. (Calnek et al., 1997). Most pathological changes are found in the subacute and chronic stages of the disease in liver and spleen; multiple white foci, severe swelling, and discoloration (Gast, 1997). Postmortem signs of pullorum disease in newly hatched chicks are those of peritonitis with generalized congestion of tissues and inflamed unabsorbed yolk sac (Gast, 2003). Longer standing infections commonly lead to typhlitis with development of necrotic cecal casts and small necrotic foci in the liver, lungs and other viscera (Manoj et al., 2015). Adult birds develop misshapen or shrunken ovaries with follicles attached by pedunculated fibrous stalks. Variant strains of *S*. Pullorum do not normally cause clinical disease or may result in mild, nonspecific signs but may lead to seroconversion.

### 2.5 Economic impact of hepatomegaly and splenomegaly syndrome

The chickens affected by these diseases exhibited various clinical signs such as growth retardation, depression, and the presence of pale combs and wattles (Moru et al., 2022). Unfortunately, these conditions can lead to significant mortality and decreased egg production, resulting in substantial economic losses for farmers (Paola-Massi et al., 2005). Marek's disease (MD) imposes both direct and indirect economic burdens on the poultry industry. Direct losses stem from hen mortality and morbidity, including factors like decreased egg production. Indirect losses are incurred through the industry-wide implementation of vaccines and control measures (Fehlar et al., 2001). In Nigeria, a nine-year study on avian neoplastic diseases highlighted that lymphoid leukosis accounted for 14.10% of total avian neoplastic disease outbreaks. Similarly, a one-year study in the poultry population of Mizoram reported a 5.88% prevalence of lymphoid leukosis, underscoring its economic impact due to mortality and performance reduction (Bhutia LD et al., 2017).

Colibacillosis is a prevalent disease in poultry flocks, affecting birds of all ages, and it has a significant economic impact on global poultry production. It stands as one of the primary causes of mortality in commercial layer and breeder chickens (Masud et al., 2012). Salmonellosis, another disease responsible for hepatomegaly and splenomegaly syndrome, also carries a substantial economic burden, particularly in the poultry industry, and has a global distribution (Rajagopal and Mini, 2013).

In summary, these diseases not only pose a health risk to poultry but also result in considerable economic losses for farmers and the poultry industry as a whole.

# CHAPTER III

# MATERIALS AND METHODS

## 3.1 Statement of the experiment

The experiment was conducted at the Department of Pathology and Parasitology, Faculty of Veterinary Medicine (FVM), Chattogram Veterinary and Animal Sciences University (CVASU), Chattogram, Bangladesh from June 2021 to January 2022.

## 3.2 Study population and study period

For identification of the possible causal agent that causes hepatomegaly and splenomegaly in commercial poultry in Bangladesh, an investigation was undertaken between January 2021 to June 2022 at Chattogram, Bangladesh. Study population includes 150 birds both broiler and layer. All the related laboratory investigations were performed at the Pathology Laboratory at the Department of Pathology and Parasitology in CVASU.

## 3.3 Collection of samples

A total of 35 samples (15 Layers, 20 Broilers) were collected during the study period. Samples consisted of the liver and spleen. Samples from each chicken were collected separately in a plastic zipper bag with proper labeling and samples for histopathological examination were kept in 10% natural buffered formalin and Buin's solutions.

## 3.4 Sample preservation

After the collection of samples, one part was kept in a zipper bag for bacteriological and viral identification. This part of the sample was preserved in a refrigerator at -20 degrees Celsius temperature. Another part of the sample is kept for histopathological examination. For preservation, two solutions are used. One is 10% natural buffer formalin and Bouin's solution. For making Bouin's solution, 750ml of aqueous saturated picric acid solution, 250ml of 40% formalin solution, and 50ml of glacial acetic acid are mixed properly. For making a 10% buffer solution 10ml buffer formalin is added with 90 ml water. 10-fold of the solution is used to preserve the deserved number of samples.

### 3.5 Histopathological procedure

For histopathological study of collected samples first permanent histopathological slides need to be prepared. For that following procedures are followed.

#### 3.5.1 Fixation of tissues

At first, collected tissue was kept in the fixative (10% buffer and Bouin's solution). For around 7 days tissue is kept in this solution. The objective was to harden the tissue so that it becomes easy to cut.

#### 3.5.2 Removal of fixative

After keeping the sample for at least 7 days tissue is kept under running water overnight in order to remove the fixative agents from the tissue.

#### 3.5.3 Dehydration

The objective of dehydration was to remove the water from the tissue. For this reason, tissue is run through a specific concentration of alcohol for a specific time. The concentration and time allowance were given below.

i. Alcohol 80% for two hours
ii. Alcohol 95% for one hour (two changes)

#### 3.5.4 Clearing

Xylene was used as a clearing reagent. The purpose of clearing is to remove the alcohol from the tissue. Tissue was run through the xylene solution for a specific time.

i) Xylene one hour (two changes)
ii) Xylene for two hours

#### 3.5.5 Impregnation

It was done to remove clearing agents. It was done by bathing in paraffin having a temperature of 56-58 degrees Celsius. Here three changes are done where tissue is kept for two hours before every change. After completing impregnation, the tissue was kept for rest overnight.

### 3.5.6 Embedding

It was done by placing the tissue in melted paraffin to make the block, which after solidification provided a firm medium for keeping all parts of the tissue intact when sections were cut.

### 3.5.7 Preparation of tissue section

For the preparation of tissue sections following processes are followed:

i) A tissue block embedded in paraffin was set in the microtome machine. Then sections were cut at 3-5 μm thickness until suitable tissue film was formed.

ii) Flim of tissue sections were placed in the warm water bath (55-58) and allowed to spread.

iii) A small amount of gelatin was added to the water bath for better adhesion of the section. to the slide.

Sections were picked up on grease-free clear slides.

### 3.5.8 Preparation of Harri's Hematoxylin Solution

▪ Hematoxylin Crystals: 5.00g
▪ 100% Alcohol: 50.0ml
▪ Ammonium alum: 100g
▪ Distilled water: 1000ml
▪ Mercuric oxide (red): 2.5g

The hematoxylin and the alum were dissolved in alcohol and in water respectively by the aid of heat. Both the solutions were thoroughly mixed just after removal from heat and boiled as soon as possible. Then mercuric oxide was added slowly after removal from heat. The solution was again heated to simmer and stopped heating when it became dark purple in color. Then the vessel was plunged into a basin of cold water and made it cool. Two to four ml of glacial acetic acid was added per 100 ml solution just before use to increase the precision of nuclear stain.

### 3.5.9 Preparation of Eosin Solution

a) 1% Stock Alcoholic Eosin

▪ Yellow eosin, water-soluble: 1g
▪ Distilled water: 20ml
▪ Dissolved and add 95% Alcohol: 80 ml

b) Working Eosin Solution

- ▪ Eosin Stock Solution: 1 part
- ▪ 80% Alcohol: 3 parts 0

Half ml of glacial acetic acid was added to each 100ml of stain and stirred to mix properly just before using.

### 3.5.10 Hematoxylin Eosin Staining Procedure

The tissue section that was taken into the slide is prepared for staining. The following procedures were followed for the staining of the slide.

- ➢ Xylene ……………… two changes each for 5 mins
- ➢ 100% alcohol……….. two changes each for 5mins
- ➢ 95% alcohol…………. 2mins
- ➢ Tap water…………….5mins
- ➢ Harris hematoxylin…….10mins
- ➢ Running tap water…….10mins
- ➢ 1% acid alcohol…….2dips
- ➢ Running tap water…...5mins
- ➢ Ammonia water………3dips
- ➢ Running tap water……10mins
- ➢ Eosin stain………...2mins
- ➢ 95% alcohol……….3mins
- ➢ 100% alcohol…….3mins
- ➢ 100% alcohol…….3mins
- ➢ Xylene……………two changes each for two mins
- ➢ After that put DPX on the stain tissue and place a cover slip on it.

The tissue is ready to study for histopathological changes. Then under the microscope in different magnifications, the study was done and find the abnormal changes in cellular levels.

### 3.6 Isolation of bacteria (*Salmonella sp and E. coli*)

At first, the sample was kept in a zipper bag at -20 degrees Celsius from here 1 gram was taken. This 1-gram sample was kept in 9ml buffer peptone water. Then the vial that contains 9 ml buffer peptone water and 1 gram sample was incubated overnight at 37 degrees Celsius temperature. After that 100-micro liter from the tube was taken into

9.9 ml RV media. Then this solution was incubated at 42 degrees Celsius temperature for overnight. Samples were picked up and streaked onto Xylose Lysine Deoxycholate agar (XLD) (Oxoid Ltd., pH: 7.4±0.2) with an inoculating loop and incubated aerobically at 37°C overnight.

For the isolation of *E. coli* from the collected samples, the sample was at first inoculated into a test tube containing buffer peptone water (BPW) (Oxoid Ltd, PH: 6.2±0.0, Basingstoke, Hampshire, UK) and incubated at 37°C overnight for primary enrichment. In the case of a liver sample, 2-3 grams of the sample after primary enrichment, the culture was streaked on MacConkey agar medium (Oxoid Ltd, PH: 7.4±0.2, Basingstoke, Hampshire, UK) and incubated at 37°C for 24 hours. Bright, pink-colored large colonies yielded on a MacConkey agar plate were suspected as the growth of *E. coli*. Such colonies were streaked onto the EMB agar plate (Merck, PH: 7.1±0.2) and incubated at 37°C for 24 hours. Based on the "green metallic sheen" colony morphology yielded on this medium was taken as the growth of *E. coli.*

### 3.7 Identification of Marek's disease virus by PCR

At first, DNA was extracted from the collected tissue (liver and spleen). Following was the process of DNA extraction from tissue samples.

Genomic DNA purification consists of two stages. The first stage was sample lysis, and the second stage is genomic DNA binding and elution.

#### 3.7.1 Sample lysis (BioLabs™)

- Tissue was cut into small pieces to ensure rapid lysis and high yields. 15mg sample was taken and placed in a 1.5ml microfuge tube.
- Then 10 microliter proteinase k and 200 microliter tissue lysis buffers was added to each sample. Mixed immediately by vertexing.
- Incubated at 56 degrees Celsius in a thermal mixer with agitation at full speed (1400rpm) until tissue pieces have completely dissolved.
- Centrifuged for 3 minutes at 12000 rpm to pellet debris. Transfer the supernatant to a fresh microfuge tube.
- Then we added 3 microliter RNase A to the lysate, vortex thoroughly, and incubate for 5 minutes with agitation at full speed.

### 3.7.2 Genomic DNA binding and elution (BioLabs™)

- First 400 microliter DNA binding buffer was added to the sample and mixed thoroughly by pulse-vertexing for 5 minutes.
- Then binding buffer mix (~600 microlitter) was transferred to a g DNA purification column pre-inserted into a collection tube, without touching the upper column area.
- Centrifuged for 3 minutes at 1000 rpm to bind g DNA and then for 1 minute at 12000 rpm to clear the membrane. Discarded the flow-through and collection tube.
- Transferred the column to a new collection tube and add 500 micro litter g DNA wash buffer. Closed the cap and inverted a few times and centrifuged immediately for 1 minute at maximum speed then discard the flow through.
- After replacing the column with the collection tube. Add 500 micro litter g DNA wash buffer and close the cap. Centrifuged immediately for 1 minute at maximum speed. Then again discarded the collection tube and flow through.
- Finally, we placed the g DNA purification column in DNase free 1.5ml microfuge tube. Added 100 microliter preheated (60 degrees Celsius) g DNA elution buffer, closed the cap and incubate at room temperature for 1 minute.
- Centrifuged for 1 minute at maximum speed to elute the g DNA.

### 3.7.3. Identification of Marek's disease virus by PCR

The PCR assay was conducted for the final detection of isolates (Kamaldeep et al., 2013) by PCR using the set of primers described in Table 1.

### 3.7.4. Primers and PCR assay

Table 1. Sequences of oligonucleotide primers used in the detection of MDV through PCR

| **Primer** | **The sequence of primer (5'-3')** | **amplified product** | **Reference** |
|---|---|---|---|
| **ICP4 F** | 5'GGATCGCCCACCACGATTA CTACC3' | 318 | (Kamaldeep et al., 2013) |
| **ICP4 R** | 5'ACTGCCTCACACAACCTCA TCTCC3' | | |

In the table 1 we used the oligonucleotide sequence of the primer for the PCR as this sequence is used in the previous study by Kamaldeep et al., 2013.

**Table 2. Composition of each reaction mixture**

| Ingredients | Amount |
|---|---|
| Thermo Scientific™ dream Taq PCR master mix | 12.5 µl |
| Forward Primer | 1 µl |
| Reverse Primer | 1 µl |
| DNA template | 5 µl |
| Deionized water (Nuclease free) | 6.5 µl |
| Total | 25 µl |

Amplification (PCR) was performed in a thermal cycler (Applied Biosystem®, 2720).

A stock of each primer with a concentration of 100pmol/µl was diluted by adding nuclease-free molecular grade water to make a 20 picomole per µl concentration to run PCR assays. PCR was done in a 25 µl total reaction volume where different volume of reagent used that is given in the table 2.

**Table 3. The cyclic conditions used for PCR**

| Serial no | steps | Time consumption |
|---|---|---|
| **1** | Initial denaturation | 94° C for 3 min |
| | Final denaturation | 94° C for 40 sec |
| **2** | Annealing | annealing at 58° C for 40 |
| | Extension | 72° C for 1 min |
| **3** | Final extension | 72° C for 5 min |
| **4** | Final holding | 4°C |
| **5** | Cycle of **2*** | 31 times |
| Adapted from (Kamaldeep et al., 2013) | | |

For a negative control master mix without any DNA template and for a positive control a previously isolated positive strain was used. PCR products (amplicons) were stored at 4°C until analyzed by electrophoresis in 1.5% agarose gel containing ethidium bromide.

### 3.7.5. Procedure of agar gel electrophoresis

- For 1.5% agarose gel, 500 mg of agarose powder and 50 ml of 1X TAE buffer were mixed thoroughly in a conical flask.
- The mixture was heated in a microwave oven until agarose was completely dissolved.
- The agarose-TAE buffer solution was then allowed to cool at room temperature.
- Gel casting was prepared by sealing the ends of the gel chamber with the appropriate casting system and placing combs in the gel tray.
- 10µl of ethidium bromide was added to the agarose-TAE buffer mixture, shaken well, and poured into the gel tray.
- The gel was then allowed to be cooled at room temperature for about 30 minutes.
- The combs were removed, and the electrophoresis chamber was filled with 1X TAE buffer to drown the casted gel.
- 4µl of PCR product and 2µl of 100bp marker (ladder) were loaded into the gel.
- The electrophoresis was run at 120V and 90mA for 20 minutes.
- Then the gel was taken to the UV transilluminator for image acquisition and analysis.

# CHAPTER IV

# RESULTS

## 4.1 Percentage of *E. Coli* isolates based on cultural properties.

Table 4. Percentage of *E. Coli* based on cultural properties.

| Production type | No. of observations | Number of positive isolates on EMB agar media | percentage |
|---|---|---|---|
| Broiler | 20 | 10 | 50% |
| Layer | 15 | 7 | 46.6% |
| Total | 35 | 17 | 48.5% |

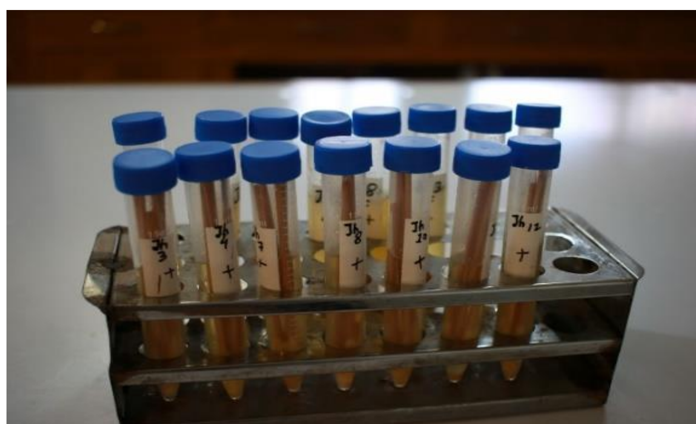

Figure 1: Growth on broth

Figure 2: Growth on MacConkey agar

Figure 3: Growth on EMB agar

In figure 1, buffer peptone water is used, opacity of this indicate growth of bacteria. In figure 2 pink color colony indicate growth of *E. coli* and in figure 3 green color metallic shin colony indicates growth of *E. coli.*

On the basis of this cultural properties total 48.5% birds were found positive for *E.coli.* Among all birds 50% broiler birds of different ages found positive for *E.coli* where we observed hepatomegaly and 46.6% layer birds found positive for *E.coli* where only hepatomegaly was observed.

**4.2 Percentage of *salmonella* isolates based on cultural properties.**

No positive characteristics growth is observed in culture.

**4.3 Percentage of ALVs based on gross and microscopic lesions.**

Table 6. Percentage of ALVs based on gross and microscopic lesions.

| Production type | No. of observations | Number of positive results | percentage |
|---|---|---|---|
| Broiler | 20 | 0 | 0 |
| Layer | 15 | 4 | 26.5% |
| Total | 35 | 4 | 11.4% |

We found nodular lesions on the liver that was enlarged. We found such kind of nodular lesions in four birds. In a study conducted by Moru et al. (2022), in case of lymphoid leukosis anatomic analysis showed that most of chickens had enlarged livers,

twice to three times bigger than normal ones. The livers were soft, mottled, and fragile, containing many miliary nodules, and hemorrhagic spots, stippled with red, yellow foci on the surface. The spleen was also mottled and enlarged, from mildly to severely. Based on this necropsy findings, no such kind of lesions were found in broiler birds. Total four-layer birds showed this type of lesions among 15 layers birds. So total 35 birds observed where liver and spleen were enlarged only 11.4% mentioned as positive for lymphoid leukosis.

### 4.4 Percentage of Marek's disease virus based on the number of positives in PCR

Table 5. Percentage of Marek's disease virus based on the number of positives in PCR

| Production type | No. of observations | Number of positive isolates on PCR | percentage |
|---|---|---|---|
| Broiler | 20 | 0 | 0 |
| Layer | 15 | 3 | 20% |
| Total | 35 | 3 | 8.5% |

We have done PCR for three suspected cases where both spleen and liver were very enlarged. We used ICP4 gene and oligonucleotide primer sequence that was used in a previous study by Kamaldeep et al., (2013). We found positive band for 318 bp that was shown in figure no 4. Based on positive result found in PCR only three layers birds among fifteen layers birds marked positive for Marek's disease.

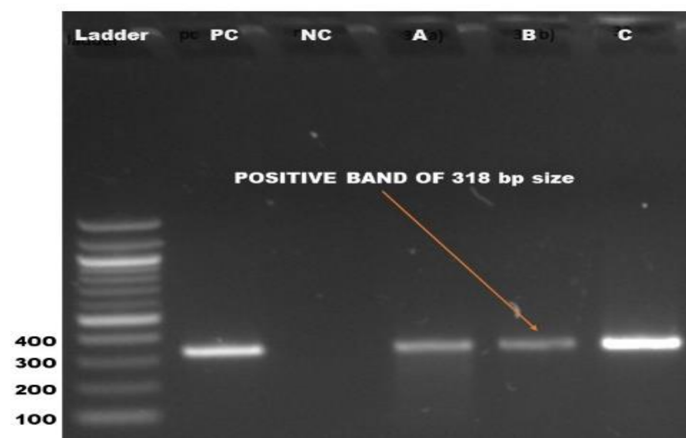


Figure 4: Amplification of ICP 4 gene in PCR

## 4.5 Gross findings of hepatomegaly and splenomegaly syndrome

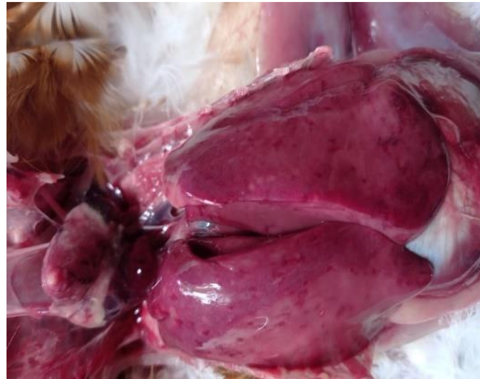

Figure 5: Enlarged liver

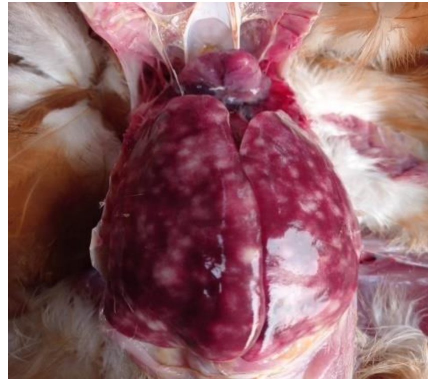

Figure 6: Nodular lesion in liver.

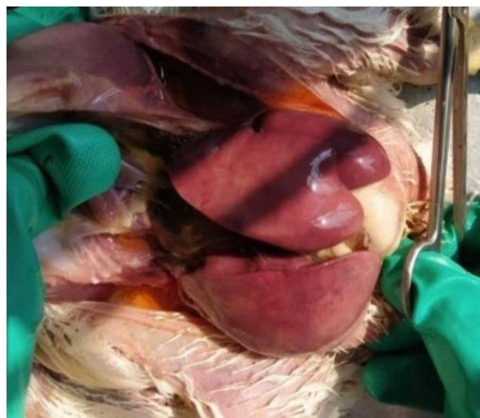

Figure 7: Congestive and enlarged liver

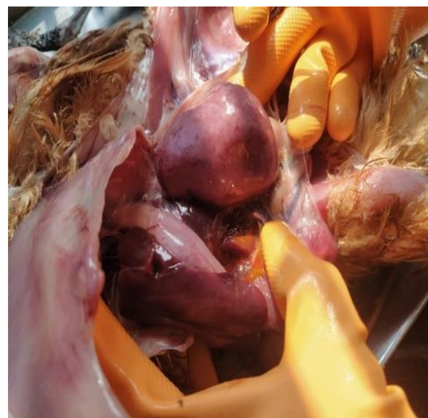

Figure 8: Enlarged spleen.

Figures 9 &10: Enlarged spleen (compared with normal sized spleen)

### 4.6 Microscopic findings of hepatomegaly and splenomegaly syndrome:

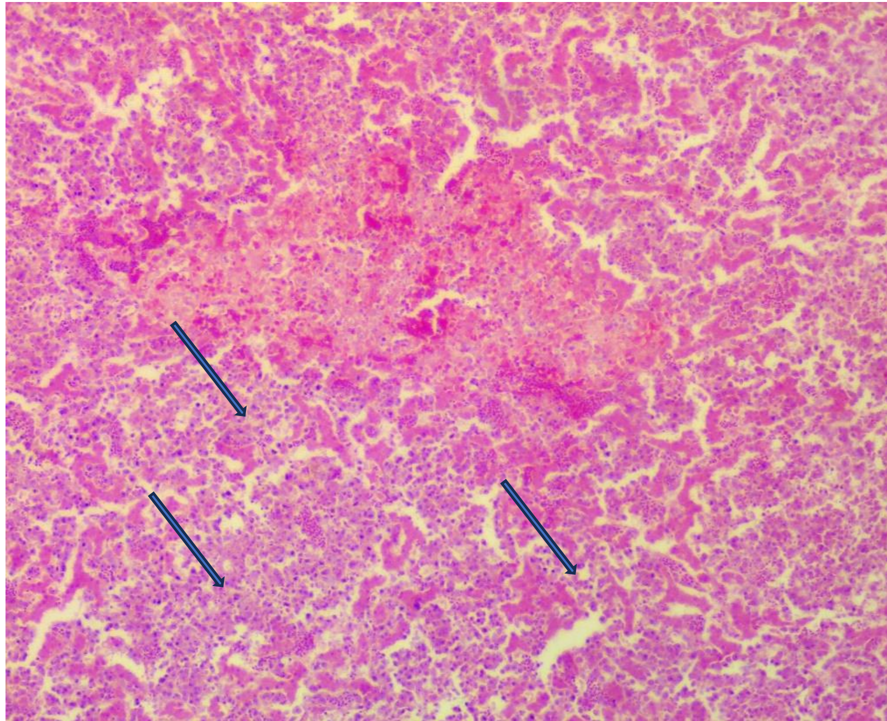

Figure 11: Accumulation of lymphoid cells in liver (Blue arrow)

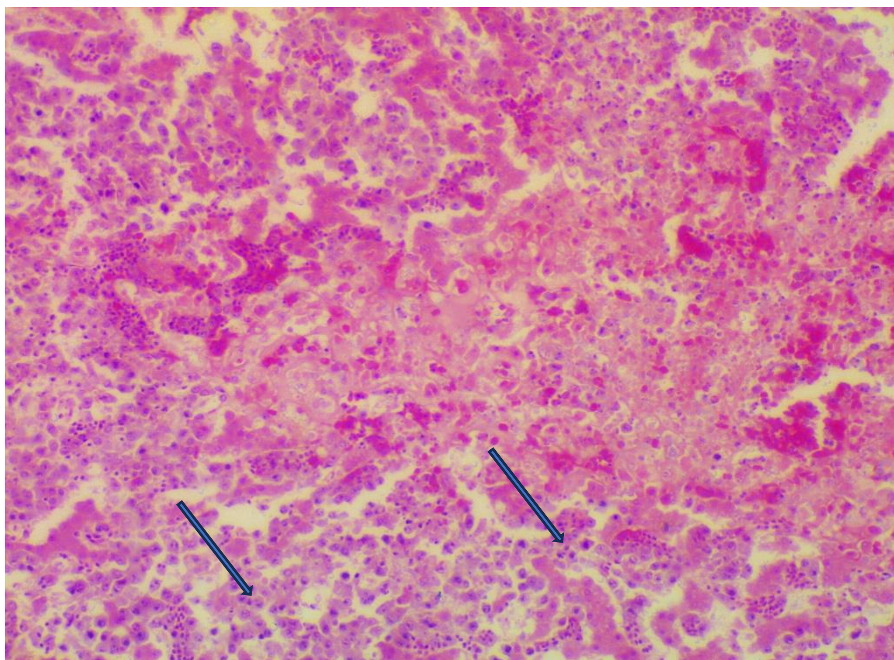

Figure 12: Accumulation of lymphoid cells in the liver (Blue arrow)

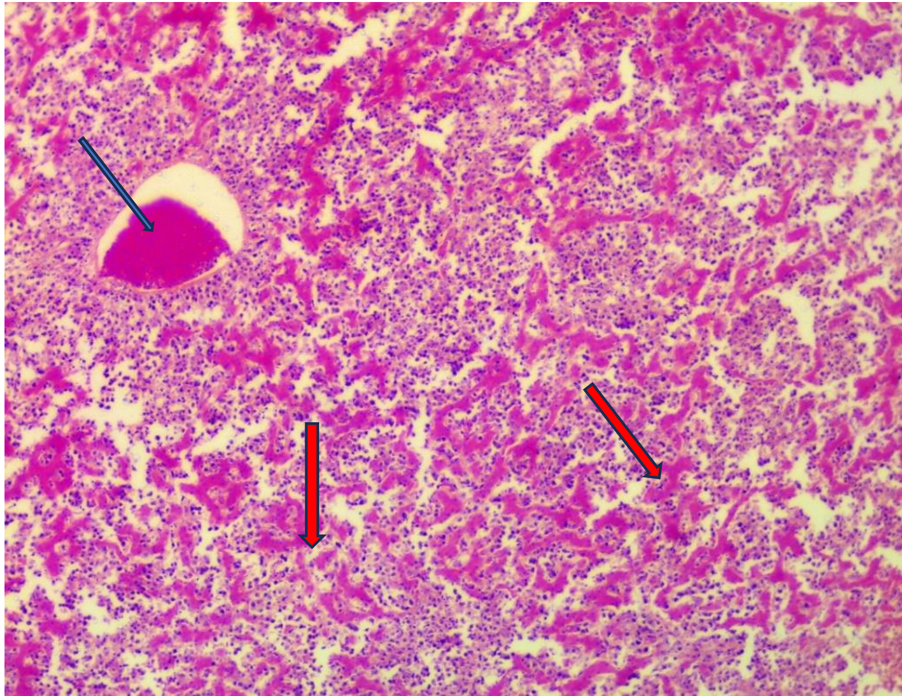

Figure 13: Severe congestion (Blue arrow) and hemorrhage in the liver (Red arrow)

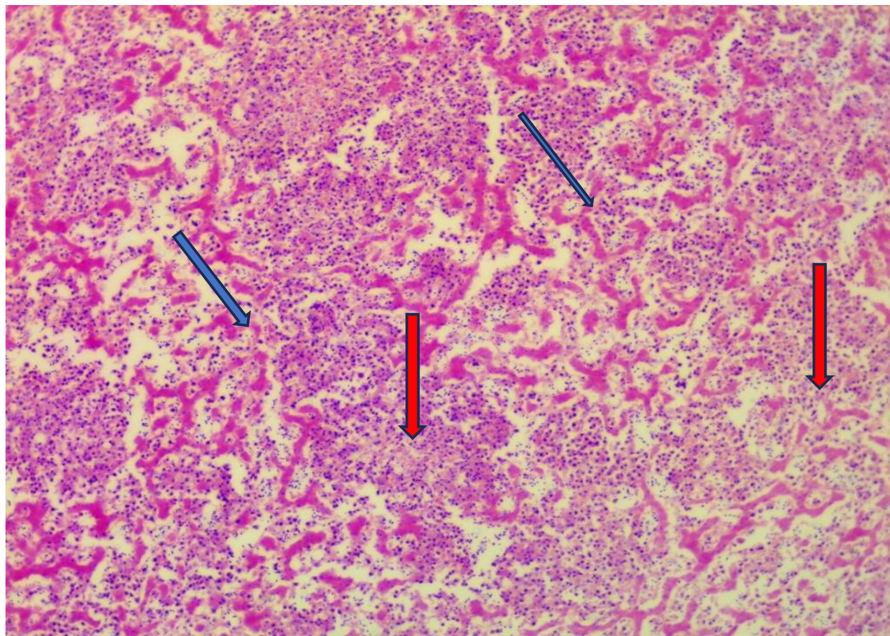

Figure 14: Dissociation of hepatic cord (Blue arrow) and accumulation of mononuclear cells (Red arrow)

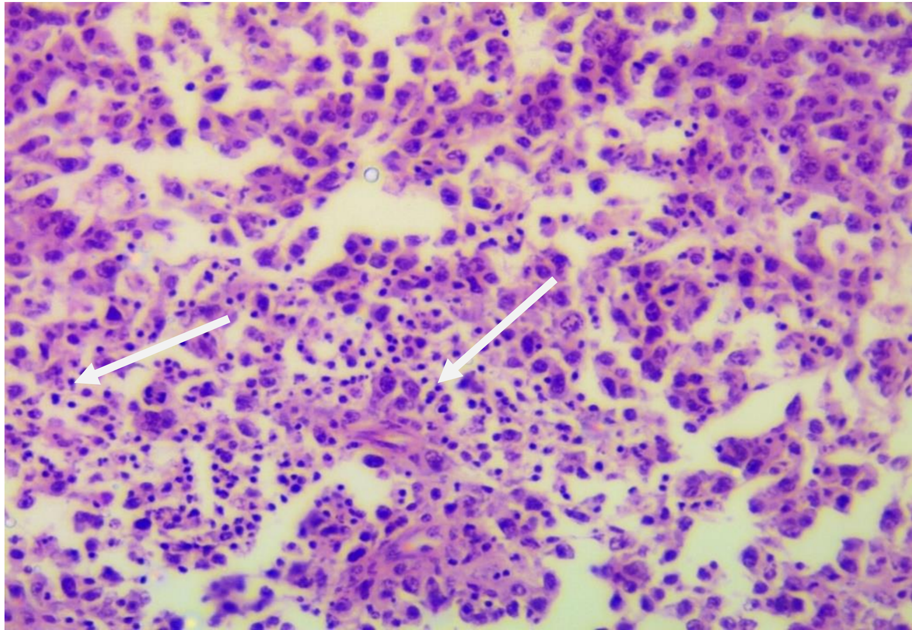

Figure 15: Lymphocytic accumulation in the liver (white arrow)

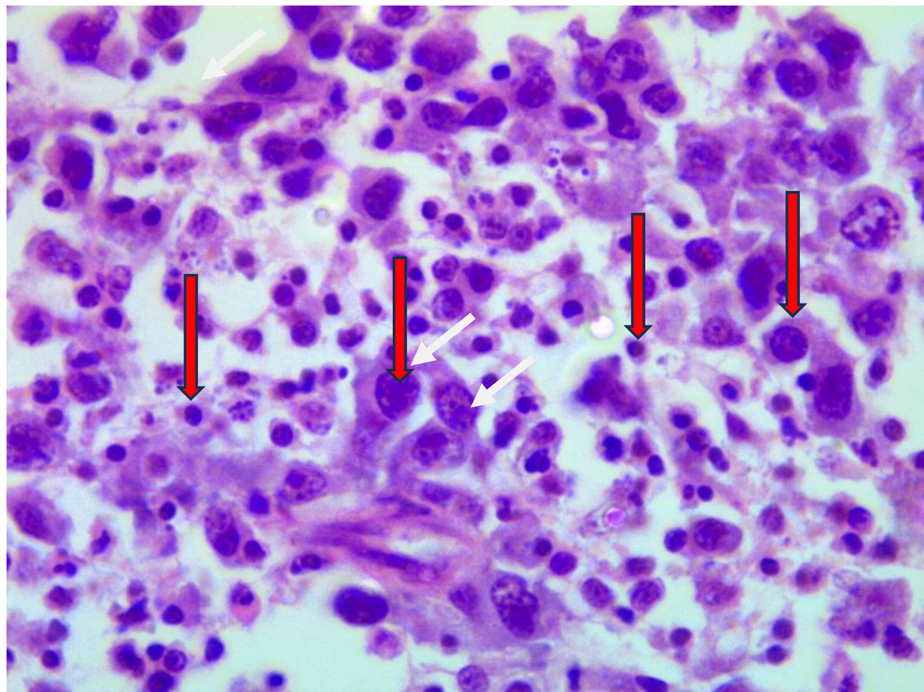

Figure 16: Accumulation of large pleomorphic cells in the liver (lymphoblastic and lymphocytic cells)

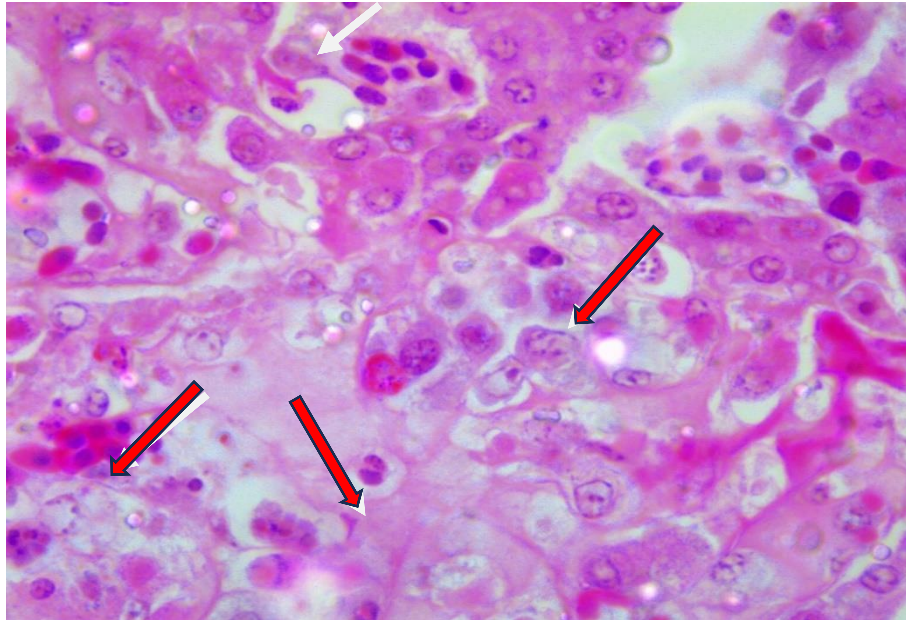

Figure 17: Infiltration of lymphocyte and macrophage in the nodule

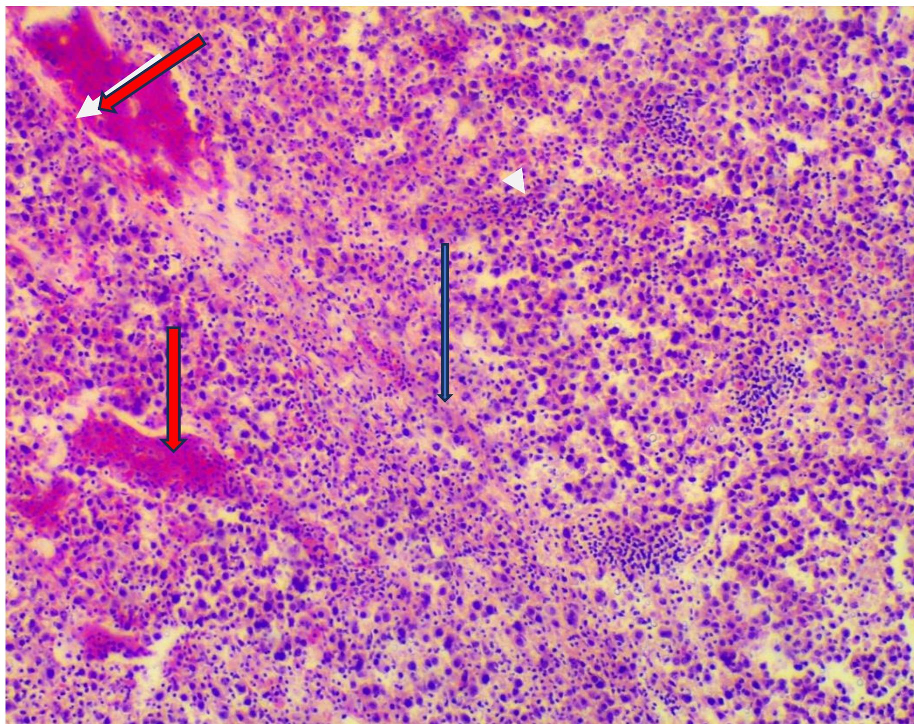

Figure 18: Congestion (Red arrow) and hemorrhage in spleen (Blue arrow)

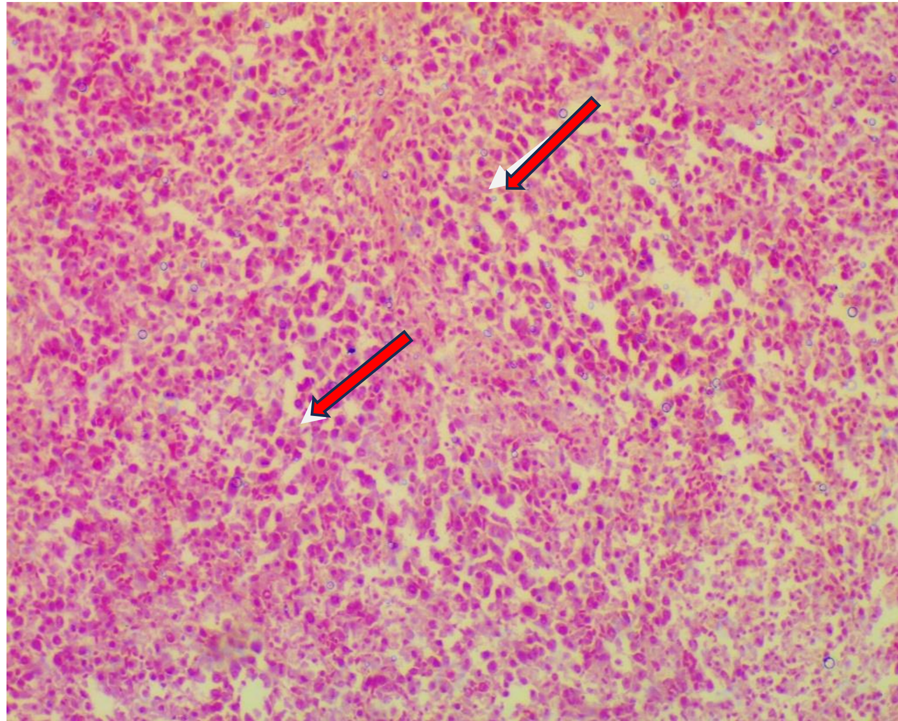

Figure 19: Proliferation of mononuclear cells and macrophage in spleen

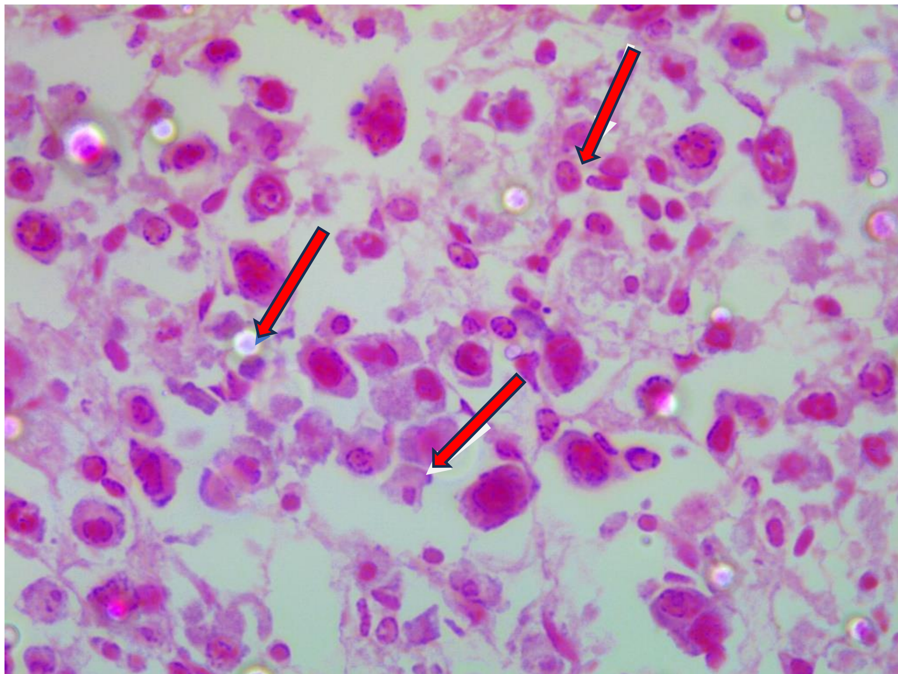

Figure 20: Pleomorphic cells accumulation in the spleen

# CHAPTER V
# DISCUSSION

Hepatomegaly and splenomegaly represent a common syndrome observed in both commercial broiler and layer chickens. Various diseases can affect different organs and systems in poultry, and in this case, the liver and spleen are vital organs that often become enlarged due to the impact of several viral and bacterial diseases. Research has indicated that viral etiology plays a predominant role in the development of these conditions (Barnes et al., 1997). Among the viruses associated with hepatomegaly and splenomegaly in commercial layers, some of the most identified ones include avian hepatitis E virus, avian leukosis virus, and Marek's disease virus (Payne and Fadly, 1997). In summary, hepatomegaly and splenomegaly are frequently observed conditions in commercial poultry, and viral infections, particularly avian hepatitis E virus, avian leukosis virus, and Marek's disease virus, are recognized as significant contributors to the development of these symptoms in commercial layer chickens.

As reported by Kabir et al. (2010), within the spectrum of poultry diseases, avian colibacillosis, primarily caused by *Escherichia coli (E. coli),* is recognized as a significant contributor to both morbidity and mortality rates. This bacterium can act as either a primary pathogen or a secondary one, and detailed postmortem examinations have revealed specific pathological characteristics. These include diffuse splenomegaly and hepatomegaly, where the spleen and liver exhibit multifocal greyish areas on their surfaces. Additionally, the kidneys in affected birds are diffusely enlarged, congested, and display a mottled pale appearance. Similarly, Saha et al. (2012) have documented the effects of Salmonella infection in birds. In cases of salmonellosis, the liver is notably enlarged, congested, and exhibits a friable texture. The coloration of the liver is bronze, and there may be white necrotic foci present.

In summary, avian colibacillosis caused by *E. coli* and salmonellosis both manifest distinct pathological changes in poultry, affecting organs like the liver, spleen, and kidneys, and can lead to severe morbidity and mortality in affected birds.

The primary objective of this study was to identify the specific causative agents responsible for the development of hepatomegaly and splenomegaly syndrome in commercial chickens. Similar studies have been conducted in various countries to understand the underlying factors contributing to this syndrome. For example, in Italy,

a study conducted in 2005 by Paola Massi aimed to investigate Big Liver and Spleen Disease (BLS) in poultry. In the Italian study, the primary focus was on identifying the actual agents behind this syndrome. The researchers conducted bacteriological examinations and isolated Escherichia coli (E. coli) strains. Additionally, virological examinations revealed the presence of avian hepatitis E virus as a potential contributor to the condition. In this study, found that 46% of the samples tested positive for colibacillosis in the culture test. This was observed in both layer and broiler chickens, and the affected birds exhibited enlarged and congested livers. These findings suggest that *E. coli* may be a significant factor contributing to hepatomegaly and splenomegaly syndrome in commercial chickens, aligning with the results of the Italian study. These research efforts collectively contribute to a better understanding of the causative agents of hepatomegaly and splenomegaly syndrome in poultry, which is essential for developing effective control and prevention strategies in the poultry industry.

In a study conducted by Nakamura et al. (1985), the researchers observed several pathological findings associated with colibacillosis in poultry. These included dark-colored swollen liver and spleen, fibrinopurulent lesions, airsacculitis, pericarditis, perihepatitis, hemorrhagic enteritis with fluid accumulation in ligated intestinal loops, diarrhea, arthritis, panophthalmitis, and salpingitis. These gross findings were indicative of the disease condition caused by Escherichia coli (E. coli) infection. Similarly, according to Surjagade et al. (2020), the gross findings characteristic of colibacillosis included the presence of fibrin covering on the surface of the liver and heart, which served as diagnostic markers for the disease. Histopathological examinations revealed multiple areas of focal necrosis, congestion, hemorrhage, edema, and fibrinous thickening in the liver, heart, and lung. These tissues also displayed mononuclear cell infiltration. The spleen exhibited similar lesions to the heart, liver, and lung, with lymphocyte depletion.

In this study, all the samples that tested positive were found to have colibacillosis, and this was associated with enlarged and congested livers, as illustrated in figures 5 and 6. Although the spleen was not enlarged, it showed signs of congestion. Microscopic examination revealed severe congestion and hemorrhage in both the liver and spleen, as depicted in figures 18 and 24. These findings align with the previous studies mentioned, providing further support for the diagnosis and understanding of colibacillosis in commercial chickens, both in layer and broiler populations. These

consistent findings contribute to our knowledge of the pathological characteristics of colibacillosis and aid in the accurate diagnosis and management of this disease in poultry. According to Saha et al. (2012), their observations of Salmonella-affected birds revealed specific pathological changes in the liver, including enlargement, congestion, friability, and a distinctive bronze coloration. Additionally, white necrotic foci were observed in the liver. Kumari et al. (2013) also reported similar findings in broiler chickens affected by Salmonella, which included lesions, the bronze coloration of the liver, splenomegaly, and necrotic foci on the liver, spleen, and heart. These birds also exhibited catarrhal enteritis with thick, slimy mucus on the mucosal surface, as well as enlarged kidneys with necrotic foci on the surface. These observations indicate that hepatomegaly and splenomegaly can occur in cases of salmonellosis.

However, in the current study, no samples were tested positive for Salmonella. There could be several reasons for this discrepancy, such as the sample size being too small to detect Salmonella or the possibility that the infection in the sampled birds was in a chronic stage, making it more challenging to detect the pathogen. Further research and a larger sample size may be necessary to investigate the presence and prevalence of Salmonella in commercial chickens in this study area.

Avian leukosis viruses (ALVs) are known to induce neoplastic conditions in chickens, commonly referred to as lymphoid leukosis. This condition is characterized by various names, including big liver disease, lymphatic leukosis, visceral lymphoma, lymphocytoma, lymphomatosis, and visceral lymphomatosis (Payne and Fadly, 1997). Typically, lymphoid leukosis becomes evident in birds around four months of age. One diagnostic feature, though not always present, is the gross nodular tumorous involvement of the bursa of Fabricius. Microscopically, key diagnostic features include the presence of uniform large lymphocytes (lymphoblasts) constituting the tumors, intrafollicular tumors within the bursa, and the tendency for tumors in other organs like the liver and spleen to grow in an expansive nodular pattern.

In the present study, we observed significant signs and lesions consistent with lymphoid leukosis in layer birds. These included weakness, diarrhea, dehydration, emaciation, anorexia, pale wattles, and abdominal enlargement. Gross lesions of emaciation and dehydrated carcasses were prominent, along with markedly enlarged liver and spleen.

Microscopically, the liver, spleen, and other visceral organs displayed the presence of aggregates of large lymphoid cells (lymphoblasts) of variable sizes. Notably, we observed a white-colored tumor on the surface of the liver, as depicted in Figures 9 and 10. Due to the proliferation of mononuclear cells, the spleen exhibited enlargement and a whitish coloration (figure 13). Microscopic examination confirmed the accumulation of lymphoblastic and lymphocytic cells in both the liver and spleen (figure 15, 19, 20, 22).

It's noteworthy that all these lesions were found exclusively in layer birds, with no broilers displaying similar pathological findings. This information contributes to our understanding of the occurrence and manifestation of lymphoid leukosis in poultry populations, particularly in layer chickens.

The observations we have cited from various studies, including Benton and Cover (1957), Biggs (1973), Panda et al. (1983), Kobayashi et al. (1986), Panneerselvam et al. (1990), Kalyani (2006), Kamaldeep (2007), Balachandran et al. (2009), Gopal et al. (2012), and Swathi et al. (2012), collectively describe gross lesions found in chickens affected by Marek's disease. These lesions typically manifest as tumor-like nodular growths in various visceral organs, including the liver, spleen, kidney, heart, proventriculus, ovary, lungs, trachea, and even the skin. In the liver, affected birds often exhibit an enlarged and friable organ with the presence of discrete grayish-white nodules, some of which may merge together to form larger nodules.

Here, we also found similar lesions in the liver and spleen, as shown in figures 11, 12, and 13. Notably, there were white-colored nodules present in the samples that tested positive for Marek's disease virus via PCR. These findings align with the previous studies we mentioned, further confirming the presence of characteristic Marek's disease-related lesions in the liver and spleen of affected chickens. This consistency in observations across different studies emphasizes the reliability of these gross lesions as indicators of Marek's disease in poultry and reinforces the importance of early diagnosis and intervention in managing the disease.

The microscopic lesions described in studies conducted by Benton and Cover (1957), Arulmozhi et al. (2011), Kamaldeep (2007), Balachandran et al. (2009), Gopal et al. (2012), and Swathi et al. (2012) provide valuable insights into the histopathological

changes associated with Marek's disease in chickens. These observations typically include infiltration of pleomorphic lymphoid cells, comprising small, medium, and large lymphocytes, lymphoblasts, and reticulum cells in the liver section, leading to the compression and obliteration of the normal organ structure. Marked enlargement of vessels in liver sections, often accompanied by the presence of tumor emboli, indicating metastasis to various organs and malignancy. In some sections, in addition to neoplastic changes, infiltration of heterophils, variable degrees of degenerative changes, necrosis, and edema were also observed. In spleen sections, a loss of clear demarcation between normal lymphocytes and neoplastic cells, with neoplastic cells being mostly perivascular. There was also noticeable thickening of blood vessels in the spleen.

Indeed, there are several other potential causes of hepatomegaly and splenomegaly in commercial chickens, including infections with fowl adenovirus and hepatitis E virus. It's important to note that this study focused primarily on identifying the causative agents behind these conditions, and we found significant evidence of colibacillosis, lymphoid leukosis, and Marek's disease.

However, it's essential to acknowledge that the poultry industry can be affected by a variety of pathogens and diseases, each with its unique clinical presentation and pathological changes. Fowl adenovirus and hepatitis E virus are known to cause liver-related issues in chickens, and their presence may not have been detected in this study due to various factors, such as sample size, geographical distribution of these pathogens, or the specific diagnostic methods used. Continued research and surveillance are crucial in understanding and managing the diverse array of diseases that can impact poultry health. This study has contributed valuable insights into the causes of hepatomegaly and splenomegaly in commercial chickens, and future investigations may explore the presence and prevalence of other pathogens like fowl adenovirus and hepatitis E virus in the poultry population.

In our study, we observed similar microscopic findings in the liver and spleen, which included the accumulation of lymphoid cells, mononuclear cells, and pleomorphic cells in the liver, as shown in figures 15, 19, 21, and 22. In the spleen, we noted the proliferation of mononuclear cells, accumulation of lymphoblastic cells, and pleomorphic cells, as depicted in figures 25, 26, and 27. These findings align with the

previous studies you mentioned, providing additional support for the diagnosis of Marek's disease in the sampled birds.

These findings are consistent with the previous studies we mentioned, further corroborating the presence of typical Marek's disease-related histopathological changes in the liver and spleen of affected chickens. This alignment of findings across different studies strengthens our understanding of the histopathological characteristics of Marek's disease, which aids in its accurate diagnosis and management in poultry populations.

# CHAPTER VI
# CONCLUSION

Hepatomegaly and splenomegaly represent significant challenges in the poultry industry, causing adverse effects on commercial chickens in the Chittagong district of Bangladesh. This study aimed to shed light on the histopathological and molecular detection of the causal agents responsible for this syndrome. The findings revealed that the incidence of hepatomegaly and splenomegaly was significantly higher in commercial layers compared to broilers in the Chattogram region. This observation underscores the importance of understanding the prevalence and impact of these conditions on different poultry types, which can inform targeted interventions and management strategies. Furthermore, the use of PCR as a rapid and reliable diagnostic tool, in addition to conventional cultural techniques, was highlighted in this study. PCR, RT-PCR can play a crucial role in confirming the causal agents behind hepatomegaly and splenomegaly swiftly and accurately. This emphasizes the importance of incorporating advanced molecular techniques into poultry health management practices. Overall, the data from this study emphasize the need for continued research and surveillance to better understand, diagnose, and manage hepatomegaly and splenomegaly in commercial poultry farming in Bangladesh. Addressing these issues is essential for minimizing economic losses and ensuring the well-being of poultry populations in the region.

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