Thesis Ref. No. _____

# LESION CHARACTERIZATION OF ORGANS AND ISOLATION OF MAREK'S DISEASE VIRUS IN CHICKENS IN DIFFERENT POULTRY FARMS OF BISHOFTU, CENTRAL ETHIOPIA

**MVSc THESIS**

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**MVSc PROGRAM IN VETERINARY PATHOLOGY**

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**JUNE, 2022**

**BISHOFTU, ETHIOPIA**

# LESION CHARACTERIZATION OF ORGANS AND ISOLATION OF MAREK'S DISEASE VIRUS IN CHICKENS IN DIFFERENT POULTRY FARMS OF BISHOFTU, CENTRAL ETHIOPIA

**MVSc THESIS**

A thesis submitted to the College of Veterinary Medicine and Agriculture of Addis Ababa University in partial fulfillment of the requirements for the degree of Master of Veterinary Science in Veterinary Pathology

By:
Takuma Fetene Duressa

June, 2022
Bishoftu, Ethiopia

**SIGNATURE SHEET**

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As MVSc research advisor, I hereby certify that I have read and evaluated this thesis prepared under my guidance by Takuma Fetene Duressa, entitled: **"*Lesion characterization of organs and isolation of Marek's disease virus in chickens in different poultry farms of Bishoftu, central Ethiopia*"**; I recommend that it can be accepted as fulfilling the thesis requirement for the Degree of Master of Veterinary Science in Veterinary Pathology.

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As members of the Examining Board of the final MSc open defense, we certify that we have read and evaluated the thesis prepared by Takuma Fetene Duressa, entitled: **"*Lesion characterization of organs and isolation of Marek's disease virus in chickens in different poultry farms of Bishoftu, central Ethiopia*"** and recommend that it be accepted as fulfilling the thesis requirement for the degree of Masters of Veterinary Science in Veterinary Pathology.

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## DEDICATION

This thesis is dedicated to my parents Fetene Duressa Obo and Damitu Mardassa Fayissa.

## STATEMENT OF THE AUTHOR

First, I declare that this thesis is my *bonafide* work and that all sources of material used for this thesis have been duly acknowledged. This thesis has been submitted in partial fulfillment of the requirements for an advanced (MVSc) degree at Addis Ababa University, College of Veterinary Medicine, and is deposited at the University/College library to be made available to borrowers under the rules of the library. I solemnly declare that this thesis is not submitted to any other institution anywhere for the award of any academic degree, diploma, or certificate. Brief quotations from this thesis are allowable without special permission provided that accurate acknowledgment of the source is made. Requests for permission for extended quotation from or reproduction of this manuscript in whole or in part may be granted by the head of the major department or the Dean of the College when in his or her judgment the proposed use of the material is in the interests of scholarship. In all other instances, however, permission must be obtained from the author.

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Date of Submission: June 16, 2022

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| AGPT | Agar Gel Precipitation Test |
| AHI | Animal Health Institute |
| ALV | Avian leukosis virus |
| CAM | Chorioallantoic membranes |
| CEF | Chicken embryo fibroblast |
| CPE | Cytopathic effect |
| CT value | cycle threshold value |
| DEF | Duck embryo fibroblast |
| Dpi | Days post infection |
| ELISA | Enzyme-linked Immunosorbent Assay |
| FAT | Fluorescent Antibody Technique |
| FFE | Feather follicle epithelium |
| FIA® | Flinders Technology Associates |
| GaHV-2 | Gallid alphaherpesvirus 2 |
| GaHV-3 | Gallid alphaherpesvirus 3 |
| GMEM | Glasgow minimum essential medium |
| HSV-1 | Herpes simplex virus type 1 |
| HVT | Herpesvirus of turkeys |
| IPT | Immunoperoxidase Test |
| IRL | Internal repeat long |
| IRS | Internal repeat short |
| LAMP | Loop mediated isothermal amplification technique |
| LL | Lymphoid leukosis |
| MD | Marek's Disease |
| MDV | Marek's disease virus |
| MeHV-1 | Meleagrid alphaherpesvirus 1 |
| MHC | Major Histocompatibility |
| PBL | Peripheral blood lymphocytes |
| PBS | Phosphate buffered saline |
| PCR | Polymerase chain reaction |
| qPCR | Quantitative real-time PCR |
| RE | Reticuloendotheliosis |

**LIST OF ABBREVIATIONS** (Continued)

| | |
|---|---|
| REV | Reticuloendotheliosis virus |
| SPF | Specific Pathogen Free |
| SPGA/EDTA | sucrose, phosphate, glutamate and albumin /ethylenediamine tetra-acetic acid |
| TP | Transient paralysis |
| TRL | Terminal repeats long |
| TRS | Terminal repeat short |
| UL | Unique long |
| US | Unique short |

## ABSTRACT

A cross sectional study was conducted on clinically diseased chickens suspected of Marek's disease (MD) virus infection on three intensive commercial poultry farms from January, 2022 to May, 2022 in Bishoftu town. Clinical and post-mortem examination was conducted on 70 chickens suspected of Marek's disease virus (MDV) infection, virus isolation on chicken embryo fibroblast (CEF) and molecular detection of Marek's disease virus using real-time PCR was conducted. In most of the examined chickens, classical signs of MD were observed while some did not show any clinical sign. Out of the 70 necropsied chicken, atrophy of the Bursa of Fabricius was observed in 44 (62.8%), 'gray eye' and skin nodules in 2 (2.85%), hepatomegaly in 38 (54.2%), splenomegaly in 16 (22.85%), ovary nodules in 2 (2.85%), kidney and lung nodules in 3 (4.28%), and heart nodules in 7 (10%) chickens. Peripheral nerve enlargement was not observed in any of the examined chickens. 65 feather follicles and 5 spleen samples were collected in viral transport media from the 70 clinically examined chickens under sterile condition. Molecular detection and confirmation of MDV was done using real-time PCR on the 70 samples and 53 (75.71%) of the feather follicle samples came out to be positive. Isolation of MDV was performed on CEF cell culture. Out of the 7 pooled samples, virus was isolated from 5 (72.42%). The isolates developed characteristic cytopathic effects of rounding of cells and plaques starting from the 5th day of 2$^{nd}$ and 3$^{rd}$ blind passage. Histopathological examination of 17 tissue samples of the sciatic nerves, liver, spleen, ovary, heart and lungs revealed heavy lymphocytic infiltration and multifocal lymphocytic nodules and also lymphocytic depletion was observed in the spleen. This study has confirmed the circulation of MDV in the study area showing clinical signs, lesions and cellular changes. Therefore, further study on the gross and histopathological lesions, isolation and molecular characterization of MDV, targeting different breeds, age group, and geographical areas on backyard and commercial poultry farms in Ethiopia is recommended.



**Key words**: *cell culture, chicken, histopathology, lesion characterization, Marek's disease, Marek's disease virus, polymerase chain reaction, virus isolation.*

# 1. INTRODUCTION

Poultry production in Ethiopia is a major source of domestic food and nutrition security, as well as a source of income for approximately 80% of Ethiopians (Asfaw *et al*., 2019). Furthermore, it has the potential to aid in the alleviation of poverty and socioeconomic inclusion of vulnerable groups such as the urban poor, women, the disabled, orphans and the unemployed. The production of eggs for hatching, sale and home consumption, as well as the production of birds for sale, processing, replacement and home consumption, are all advantages of poultry in Ethiopia (Ebsa *et al*., 2019).

In Ethiopia, poultry production is divided into three categories: traditional, small-scale and large-scale commercial farms. The former is based on native chicken varieties, but the latter two production systems are distinguished by exotic chicken lines that are more carefully controlled (Duguma *et al*., 2005). Local ecotypes are the most common chicken breeds in Ethiopia, with a wide range of body posture, plumage, color, comb type and production (Halima, 2007). However, poultry productivity is hampered by a number of infectious and parasitic illnesses. Diseases have significantly hampered the growth, commercialization, profitability and sustainability of chicken economic activities across Ethiopia (Asfaw *et al*., 2019).

Poultry diseases have a variety of negative economic and social consequences. Their incidence is influenced by a variety of factors, including climate, population density, management strategies and immunization status (Asfaw and Ameni, 2021). They lead to significant chicken mortality and morbidity, high pharmaceutical costs, production and market losses and can constitute a public health concern through zoonoses (Wubet *et al*., 2019). Along with other viral infections such as Newcastle disease, infectious bronchitis and infectious bursitis, Marek's disease (MD) is one of the diseases with the largest economic impact in modern poultry production globally. Marek's disease virus (MDV) infects chickens all throughout the world and the virus is found in a significant proportion of flocks (Bertzbach *et al*., 2020).

MD diagnosis is a bit complex due to avian leukosis viruses (ALV) and reticuloendotheliosis virus (REV) viruses inducing similar tumours and neoplasia to MDV-induced ones. In order to diagnose poultry lymphomas, a variety of criteria are routinely used. MD lymphomas tend to show earlier than retrovirus-induced lymphomas, therefore epidemiological parameters, particularly the age of infected chickens, can help with the diagnosis. Although this is true in some circumstances, the age criterion is not absolute because MD lymphomas can develop in older chicks, and non-bursal lymphomas caused by REV can develop as early as 6 weeks of age. In most situations, diagnostic factors, such as the distribution of lesions and the histological properties of tumor cells, are used to make the diagnosis. Pathological criteria, on the other hand, are not always sufficient. Both ALV and REV can cause tumors in the bursa with the exact same features. MDV and REV both have the ability to cause nerve lesions. Although not frequently used, tumor cell type and molecular characterization of integrated retrovirus are useful diagnostic criteria. The cell type (B cells or T cells) will be used to distinguish MD lymphomas from ALV-induced lymphomas and REV-induced bursal lymphomas. By far, the most useful technique to distinguish between these diseases is the use of serotype-specific diagnostic technique using polymerase chain reaction (Gimeno *et al*., 2005).

Previously there were studies that have been conducted in parts of Ethiopia that managed the isolation and sequencing of MDV serotypes (Lobago and Woldemeskel, 2004; Duguma *et al*., 2005; Demeke *et al.*, 2017; Yimer *et al.*, 2021). An outbreak investigation on a farm by Lobago and Woldemeskel (2004) managed to observe and categorize the gross and microscopic lesions in central Ethiopia. Demeke *et al.* (2017) have managed isolation and molecular analysis of MDV isolates circulating in chicken population in the central part of Ethiopia where commercial farms are populated whereas gross and microscopic examinations as well as virological and serological tests have been done by Duguma *et al*. (2005). An experimental investigation was conducted to determine the pathogenicity of field isolates against Marek's disease in antibody-free chicks and assess the protective efficacy of the Marek's disease vaccination by Yimer *et al.* (2021). However, further molecular, gross and histopathological lesion characterization of MD is mandatory. Therefore, the objectives of this study were as follows:

- ✓ To isolate the locally circulating strains of MDV in selected farms of Bishoftu.
- ✓ To characterize the gross and histopathologic lesions of Marek's disease.

## 2. LITERATURE REVIEW

### 2.1. History of Marek's Disease

Marek's disease is a highly contagious malignant lymphoma of chickens that causes lymphoid infiltrations in peripheral nerves, visceral organs, eye, muscle and skin, causing considerable economic losses to the global chicken industry. MD was named after Joseph Marek, a Hungarian veterinarian who originally diagnosed the disease in four adult male chickens in 1907 (Marek, 1907).

The condition, which is marked by paralysis of the legs and wings, is called "polyneuritis" or "neuritis interstitialis" by Joseph Marek. The sacral plexuses and spinal cords were thicker on the surface and several of these nerves had mononuclear infiltration under the microscope. The sickness was first documented in America as "paralysis of the domestic fowl" (Kaup, 1921).

A similar illness known as "neuromyelitis gallinarium" was described in the Netherlands shortly after (Van der Walle and Winkler-Junius, 1924). The sickness was referred to as "chicken paralysis", "range paralysis", or "Marek's paralysis" at the time. Mononuclear infiltration was identified in visceral organs in 10% of chickens that were paralyzed, in addition to peripheral nerves (Pappenheimer *et al*., 1929).

Tumours in the liver, kidneys, lungs, adrenals, muscle and most commonly in the ovary were also discovered and this disorder was named "Neurolymphomatosis gallinarium". Because of the lymphoid tumours and the difficulties in distinguishing the two diseases, lymphoid leukosis, another neoplastic condition caused by the avian leukosis virus, was frequently misdiagnosed (Ellermann, 1922). The disease can be separated from lymphoid leukosis based on susceptibility, organs affected and histopathogenesis and a new term, Marek's disease, has been proposed. It was later determined that these two diseases are distinct and are caused by two different pathogens (Biggs and Payne, 1967).

## 2.2. Aetiology of Marek's Disease

Marek's disease is a lymphoproliferative illness that causes lymphoid infiltration in the peripheral nerves and the development of lymphoid tumours in the viscera in chickens. MD is a lymphoproliferative disease of the poultry that causes clinical disease, increased mortality and reduced growth, as well as subclinical immunosuppression, which can lead to the exacerbation of other diseases and a reduction in vaccine immunity (Mescolini *et al*., 2020).

Marek's disease virus is an avian herpesvirus with a genomic structure that classifies it as an alphaherpesvirus (Buckmaster *et al*., 1988). The virus belongs to the Mardivirus genus and the Alphaherpesvirinae subfamily. It consists of three viral species: Gallid alphaherpesvirus 2 (GaHV-2) (etiological agent of MD), Gallid alphaherpesvirus 3 (GaHV-3) and Meleagrid alphaherpesvirus 1 (MeHV-1) or Herpesvirus of Turkeys (HVT), according to the most recent nomenclature (International Committee on Taxonomy of Viruses, 2017).

Both inoculated and uninoculated contact chickens acquired MD after being inoculated with blood and tumour cell suspensions collected from infected birds, demonstrating that the disease is easily transmissible. These and other studies demonstrated that the MD aetiological agent is strongly cell-associated, since cell-free suspension failed to generate a cytopathic effect in cell culture or disease in chickens (Baigent and Davison, 2004).

## 2.3. Marek's Disease Virus

### *2.3.1. Virus morphology*

Many ultrastructural investigations on the morphology and morphogenesis of MDV and HVT have been summarized by Payne *et al*. (1976), Kato and Hirai (1985) and Schat (1985). In general, viruses of all three serotypes have properties that are similar to those of other herpesviruses, according to these investigations. Because particles are uncommon in chicken tissues other than the feather follicle epithelium (FFE), most research has relied on infected cell cultures. Cell culture studies have almost exclusively been with serotype-I MDV or HVT infections (Biggs, 2000).

MDV and HVT have comparable morphology and morphogenesis in general. Their structure is typical of herpesviruses. The unenveloped virion measures approximately 100 nm in diameter and has 162 hollow capsomeres in negatively stained specimens. The capsomeres are approximately 6 x l0 nm in size, with a 10 nm centre-to-centre gap between neighbouring capsomeres. Due to the lack of stiffness in the envelope, the diameter of encapsulated particles fluctuates greatly. For example: encapsulated particles in negatively stained preparations of lysed FFE ranged in size from 273 to 400 nm (Nazerian, 1973).

Thin slices of infected cell cultures examined under an electron microscope reveal bare particles measuring 85-100 nm in the nucleus, which can seem hexagonal and, less frequently, tiny ring-shaped structures measuring 35 nm in diameter, which have long been associated with herpesviruses (Okado *et al*., 1972).

The nucleoid is 50-60 nm in diameter and is shaped like a torus that is oriented at right angles to and revolves around a less electron-opaque cylindrical mass. This explains the nucleoid's varied shape in sections, which is dependent on the angle of section, as well as the particle's developmental stage. The nucleoid in some HVT particles appears to have an electron-lucent cross look. In the perinuclear space or nuclear vesicles, enveloped particles of 130 to 160 nm in diameter are observed more seldom. In the cytoplasm, both naked and enveloped particles can be detected, however they are found less frequently than those in the nucleus (Biggs, 2000).

*2.3.2. Serotype and pathotype*

MDV and related herpesviruses have been divided into three serotypes. All pathogenic or carcinogenic strains of these viruses, as well as the low pathogenic vaccination strain CVI988 (Rispens strain), are classified as serotype 1. Serotype 2 vaccination strains of the MDV group obtained from chickens are naturally attenuated. Turkey herpesvirus (HVT), a nononcogenic MDV-related virus isolated from turkeys, belongs to serotype 3 (Table 1) (Pejović *et al*., 2007).

New pathotypes have emerged, showing that MDV is continuing to evolve toward increased virulence (Venugopal *et al*., 2001). Serotype 1 MDV strains can be divided into four pathotypes: mild (m), virulent (v), very virulent (vv) and very virulent plus (vv+). The rise in MDV virulence during the last 50 years has been significant. The transition from mMDV to vMDV strains in the late 1950s, the transition from vMDV to vvMDV in the late 1970s and most recently, the appearance of the putative vv+ MDV in the early 1990s all resulted in the potential for greater disease losses, which persisted until the introduction of a more effective vaccine. The potential to generate disease in chickens inoculated with progressively effective vaccinations is the basic basis for pathotype classification (Witter *et al*., 2005).

**Table 1:** Classification of MDV serotypes and their representative strains (Shambhu *et al.,* 2012)

| MDV serotypes | **Pathotype or strains** |
|---|---|
| Serotype 1<br>**Pathogenic or oncogenic strains as well as attenuated strain of these viruses)** | Very virulent plus (vv+): 648A<br>Very virulent (vv): Md/5,Md/11, Ala-8, RB-1B<br>Virulent (v): HPRS-16, JM GA<br>Mild (m) virulent: HPRS-B14, Conn A<br>Weakly virulent: CU-2, CVI-988 |
| Serotype 2<br>**(Naturally non-pathogenic, Nononcogenic or avirulent strains)** | SB-1, HPRS-24, 301B/1, HN-1 |
| Serotype 3<br>**(Naturally avirulent strains, nononcogenic)** | HVT(FC126, PB1)<br>(Herpes virus of Turkey) |

*2.3.3. Genome and its organization*

The MDV genome is a large, linear, double-stranded DNA with a buoyant density of 1.715 gm/cm$^2$ and belongs to the class E genome organization, which is identical to that of herpes simplex virus type 1 (HSV-1). It is made up of two distinct DNA segments, a

unique long (UL) and unique short (US), bordered by massive inverted repetitive sequences (Figure. 1). Terminal repeat long (TRL) and internal repeat long (IRL) are the names given to the flanking DNA sequences of UL, whereas terminal repeat short (TRS) and internal repeat short (IRS) are given to the flanking DNA sequences of US (Jarosinski *et al.*, 2006).

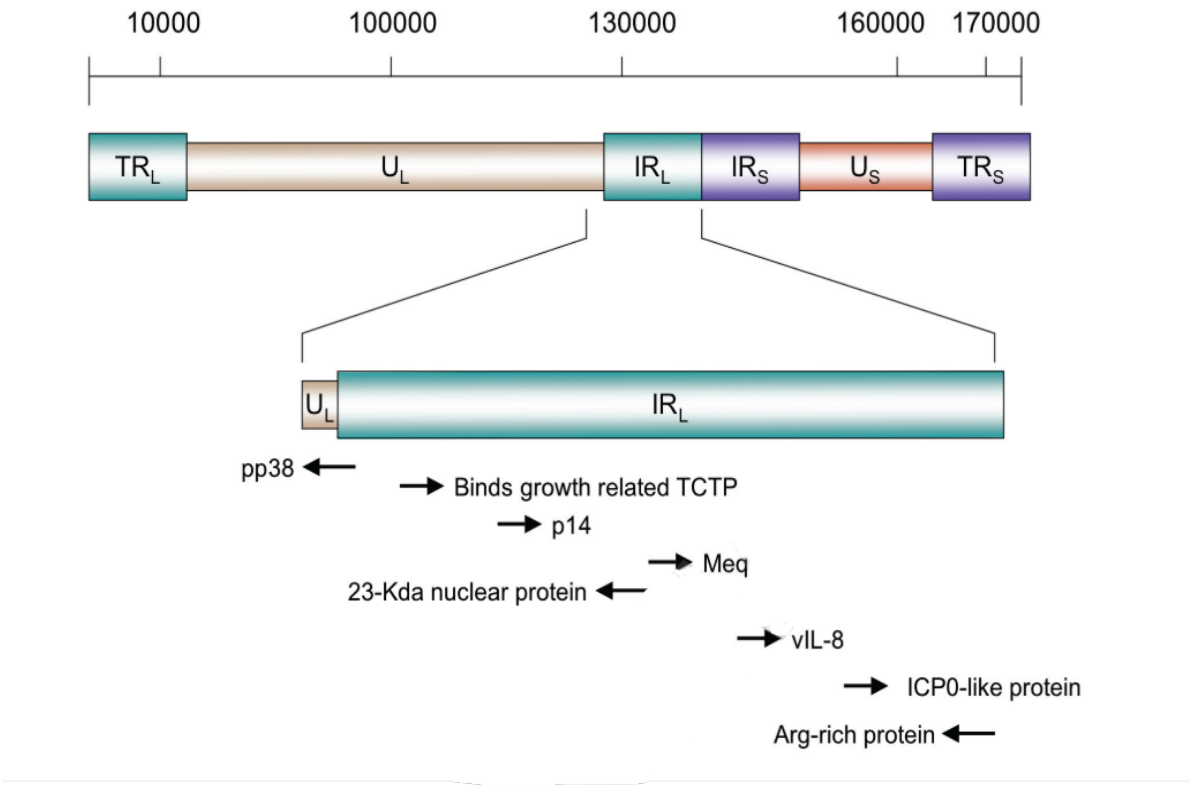



**Figure 1**: MDV genome (Kato and Hirai, 1985)

*2.3.4. Pathogenesis*

Marek's disease has a complicated aetiology, with infection spreading via the respiratory system after inhaling virus-infected chicken house dust. After an early cytolytic infection mostly of B-lymphocytes in the bursa, spleen and thymus, the virus infects activated T-lymphocytes, primarily of the CD4+ subtype, 3 to 5 days later. At 6 to 7 days after infection, the infection in T-lymphocytes becomes latent and the virus is transmitted throughout the body by infected lymphocytes, which persist as a cell-associated viremia. Approximately ten days after infection, a secondary cytolytic infection occurs in the FFE,

from which infectious cell-free virus is generated and released into the environment in feather debris and dander (Murphy *et al*., 1999).
T-lymphocytes that have recently been infected are altered, resulting in lymphomatous tumours in visceral organs. CD4+ T cells are the most common target cells for transformation in natural infections, although the virus can also transform CD8+ T-cells (Venugopal *et al*., 2001).

2.3.3.1 Virus infection with oncogenic (Serotype-I) virus

Infected chickens can carry the Marek's disease virus in almost all of their body tissues. MDV infection can be separated into four stages that lead to lymphoma formation: (I) early cytolytic, (2) latent, (3) late cytolytic and (4) transforming. These are not always discrete phases, although they are essentially sequential. The first two stages are separated by a sharp line and latent infection in certain cell types is required for transformation. However, as lymphomas progress, both transforming and latent infections may coexist with cytolytic infections in various cell populations. With phase-3 cytolytic infection, permanent immunosuppression develops concurrently (Calnek, 2001).

Following inhalation of infectious cell-free MDV from a contaminated environment, the first event, i.e., the initial infection of the chicken, occurs via the respiratory tract. The significance of the lung as a first infection location is unclear, as evidence of infection of parenchymal cells as a first event is limited at best. The lung, on the other hand, is thought to be the site where phagocytic cells pick up the virus and transport it to lymphoid organs such the bursa of Fabricius, the thymus and the spleen. Access to lymphocytes for MDV appears to be mediated by splenic ellipsoid-associated reticulum cells in blood vessel walls at these areas. A productive, cytolytic infection develops in these organs, which is most noticeable between 3 and 6 days post infection (dpi) (Calnek, 2001)

The infection in lymphoid organs transitions from productive to latent at 7-8 dpi, or occasionally somewhat later and a "viremia", or latent infection of peripheral blood lymphocytes (PBL), can be identified. At about 7 dpi, there is a transitory immunosuppression, which appears to be due to macrophage functions, as well as a transient hyperplasia in the spleen. Extrinsic factors associated with immune responses that become visible at about 6-7 dpi appear to be the most likely cause of latency, although the

lymphocyte subset may also play a role, given that most cytolytically infected cells are B cells and most latently infected cells are T cells (Gimeno, 2014).

Only the feather follicle epithelium produces a virus that is infectious in a cell-free condition, out of all cytolytically infected tissues. Although electron microscopy may reveal a few enveloped virus particles in other tissues, damaged cells provide no infectious cell-free virus. As a result, the feather follicle is one of a kind in terms of its epizootiologic significance as the source of virus that can transfer from bird to bird. Virus is commonly linked with desquamated, keratinized FFE cells that are shed with molted feathers and dander, polluting the environment and completing the infection cycle. Regardless of whether the bird develops MD, latent infection of PBL and splenic lymphocytes, as well as productive infection of the FFE, are lifetime events (Jarosinski, 2017).

Microscopic and gross lesions can be visible in a variety of locations, including lymphoid organs, visceral organs, muscle, skin, the eye, peripheral nerves and the brain, starting as early as 12-14 days after infection and lasting for weeks or months. Under the correct circumstances, even at 8-10 dpi, there could be an acute degenerative illness or a transitory paralysis. After 2 weeks or more, the development of lymphoma is usually accompanied by long-term immunosuppression that affects both humoral and cell-mediated immunity (Calnek, 2001).

#### 2.3.3.2 Virus infection with nononcogenic MDV and HVT

They have a similar basic pattern of tissue and organ infection to oncogenic serotype-I MDV, but there are some key distinctions. The most notable difference in chickens infected with HVT or attenuated MDV is the complete absence of productive, cytolytic infection in lymphoid organs or other tissues, although virus may be extracted from latently infected cells in lymphoid organs or blood. Serotype-2 viruses, on the other hand, cause at least a low level of cytolytic infection of lymphoid organs during the early infection period, but the infection thereafter becomes latent, similar to oncogenic viruses (Lin *et al*., 1991).

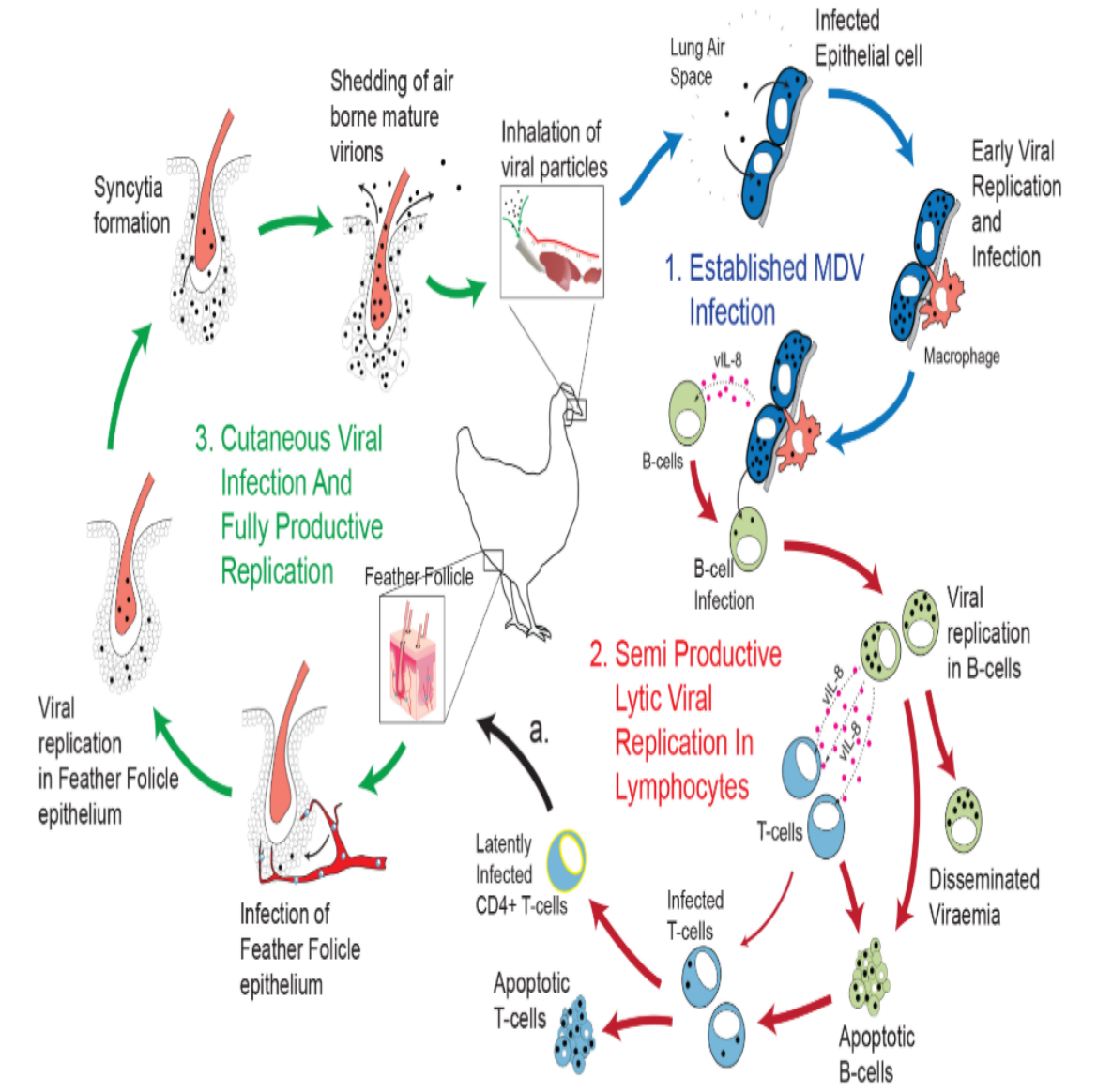



**Figure 2:** Model of the MDV infectious life cycle (Boodhoo *et al*., 2016)**.**

2.3.3.3 Transformation

The interaction of MDV with the host cell is thought to be the final cause of neoplastic transformation of latently infected lymphocytes into lymphoblastoid tumour cells. Although the spleen is most likely the primary location of converted cell proliferation, it cannot be the exclusive source of transformable target cells, as splenectomized birds still acquire neoplastic MD lesions. Three weeks after infection, T-dependent sections of the spleen become hyperplastic and diffusely dispersed T cells, thought to be progenitors of neoplastically altered cells, are found throughout the spleen (Jarosinski et al., 2006).

*2.3.5. Transmission*

Marek's disease virus is shed in dead skin and feather follicle epithelial cells, where enveloped infectious virions egress from the body that contribute to the dust found in chicken houses. This disease is spread horizontally, but it is not spread vertically from chicken to egg. The Marek's disease virus spreads by indirect contact and can remain contagious for months in habitats contaminated by infected birds. The FFE, the only place where completely productive infection and release of cell-free MDV occurs, is the source of infectious cell-free virus. Virus is shed through dead stratified epithelium cell debris, as well as moulted feathers with infected cells attached. Other chickens can inhale MDV-infected poultry dander and dust (Boodhoo *et al*., 2016).

## 2.4. Clinical Signs and Diagnosis of Marek's Disease

Clinically, there are three types of Marek's disease: classical MD, acute MD and transient paralysis. Classic MD refers to Marek's original neurological description of the disease in 1907 (Marek, 1907). The most common clinical symptom is paralysis, which is produced by lymphoid infiltration and demyelination of the peripheral nerves. Multiple and diffuse lymphomatous tumours in the visceral organs are present in the acute form. It's difficult to tell the difference between classical and acute MD. Tumours can occur in the classic disease, particularly in the ovary and nerve infiltration is common in acute conditions (Powell, 1986).

*Classical MD*: The mortality rate varies, but it rarely exceeds 10-15%. In some situations, death is limited to a few weeks, whereas in others, it happens infrequently across many months. The symptoms are determined by which peripheral nerves are damaged. The brachial and sciatic nerves are frequently involved, resulting in gradual spastic paralysis of the wings and legs. Birds that have been severely injured are unable to stand and some may lie in a distinctive position with one leg stretched forward and the other clutched behind them. Torticollis can occur when the cervical nerves are compromised and respiratory problems can occur if the vagus and intercostal nerves are impacted. Impaction, diarrhoea and weight loss may occur when the nerves to the intestinal system are damaged. The time between the development of symptoms and death might range from a few days to many weeks; in rare cases, birds can recover (OIE, 2018).

*Acute MD*: This type of MD has a substantially higher mortality rate than the conventional variant. In unvaccinated flocks, an incidence of 10-30% is not uncommon and outbreaks affecting up to 70% of the flock are possible. Mortality may spike for a few weeks and then drop or it may remain stable for several months. Many birds die suddenly without warning, while others appear despondent before passing away and some show paralytic symptoms similar to those found in the classic form; paralysis happens more frequently in birds dying late in an outbreak within a flock. Both classical and acute MD have pathological lesions that involve the proliferation and infiltration of malignantly transformed T lymphoblasts into normal tissues, including peripheral nerves in the classical form and visceral organs, including the gonads, liver, heart, kidneys and proventriculus in the acute form (Fenner *et al*., 2011).

*Transient paralysis (TP)*: MDV has been identified as the cause of relatively uncommon encephalitis in young chickens that is characterized by rapid paralysis, mainly of the neck and legs, which lasts just 3 days and is followed by recovery. At approximately 5 weeks of age, clinical signs may present in few or as many as 50% of the flock. Older, sexually mature fowl are occasionally impacted. Recovered chickens frequently succumb to clinical manifestations of MD a few weeks later. The encephalitic rather than lymphoproliferative abnormalities of transitory paralysis include perivascular lymphocyte cuffing in the cerebellum and brain stem, lymphocytic meningitis and antigen-antibody complex deposition in the meninges (Gimeno, 2014).

Marek's disease is diagnosed using gross and microscopic lesions and MDV strains can be distinguished using lesion scores based on lesions in the nerve and viscera. Polymerase chain reaction (PCR) has been developed as an additional serotype-specific diagnostic technique (Jayalakshmi *et al*., 2016).

### *2.4.1. Conventional Marek's disease diagnosis*

Age, clinical signs, history and gross and microscopic lesions are used to make a primary diagnosis. MD is easier to diagnose in chickens younger than 14 weeks of age in general. When tumours appear in older birds without bursal tumours, MD is the most likely diagnosis. Grossly, the condition manifests itself in the form of paralysis of the legs, wings and neck, as well as tumour nodules in the visceral organs, depending on the tissue or

organ involved. ‘Gray eye’ (iris) or irregular pupil, vision impairment, blindness, skin lesions and immunosuppression are some of the other signs to look for (Shambhu *et al.,* 2012).

Microscopically, mononuclear cell infiltration has been found in one or more of the following tissues: peripheral nerves, gonads, lymphoid organs, iris, muscle, skin and other visceral organs. Virus isolation and identification in susceptible (recently hatched) chicks, embryonated eggs and tissue cultures are used in the laboratory. MDV can be obtained in chicken embryos via the yolk sac pathway (4-5 days embryo) and then examined for ‘pock lesions’ (whitish raised nodules) on the 18th day of incubation by looking at their chorioallantoic membranes (CAM) (whitish raised nodules). Muscle atrophy and curling are also seen in infected embryos (Kataria *et al.,* 2005).

Duck embryo fibroblast (DEF) and chicken kidney cell culture systems can also be used to isolate MDV. Cytopathic effects (CPEs) such as plaque development are detected in cell culture after 5-14 days. Infected cells have intranuclear inclusion bodies that can be seen. Specific monoclonal antibodies can be used to confirm serotypes. The Agar Gel Precipitation Test (AGPT), Fluorescent Antibody Technique (FAT), Immunoperoxidase Test (IPT) and Enzyme-linked Immunosorbent Assay (ELISA) can all be used to detect viral antigen in feather tips, follicle epithelium and infected lymphoid tissue. MDV proteins especially the *meq* oncoprotein which is consistently expressed in all MD tumours can be effectively demonstrated using immunohistochemistry (Schat and Nair, 2008).

### *2.4.2 Recent advances in Marek’s disease diagnosis*

#### 2.4.2.1. Polymerase chain reaction (PCR)

The full length genomic sequences of MDV 1 (GenBank accession numbers: strainMd5; AF243438, GA; AFl47806, Mdl 1; AY510475, CVI988; DQ530348), MDV2 (GenBank accession numbers: strain SB-1; HQ840738, HPRS24; AB049735 and MDV 3 (GenBank accession number: strain FC126; AF291866) are available now. Serotype-specific detection of MDV is possible using PCR-based diagnostic methods. Differentiation of oncogenic and nononcogenic strains of MDV serotype and MDV vaccine strains of serotypes 2 and 3 are enabled by PCR tests (Izumiya *et al.,* 2001)

*Multiplex PCR:* Multiplex PCR has been established to detect MDV 1, avian leukosis virus and reticuloendotheliosis virus in tumour tissues of naturally infected chickens and turkeys (Gopal *et al*. 2012).

*Nested PCR***:** Nested PCR has been established to detect the MDV 1 *meq* oncogene in infected spleen cells, feather tips and peripheral blood mononuclear cells (Lee *et al.,* 2000).

*Quantitative real-time PCR (qPCR):* For simultaneous detection and quantification of viral load in clinical samples or infected tissues, qPCR to quantify MDV genome copies has been described. Because MDV 1 is so common, quantification in suspected clinical samples will be more useful than simple PCR detection (Islam *et al.,* 2004).

2.4.2.2. Loop-mediated isothermal amplification technique (LAMP)

Recently, a LAMP technique for detecting the MDV *meq* gene in affected bird feathers was developed. When compared to conventional PCR, the LAMP test required 100 times fewer copies to detect MDV and the detection time was less than sixty minutes (Wei *et al*., 2012). The LAMP approach employs three sets of primers that bind to six distinct sequences, increasing specificity. The reaction is carried out at isothermal temperatures and the results may be seen with the naked eye (Goto *et al.,* 2009).

*Use of FTA filter cards for sample collection:* Filter cards from Flinders Technology Associates (FTA®) are now being used in the field to collect, store and transport clinical samples for a variety of purposes. The FTA cards have been used to quantify MDV DNA to diagnose MD and to track the effectiveness of MD vaccinations. Samples of blood, solid tumours and feather pulp collected in FTA paper have been successfully used for the detection and quantitation of the MDV genome by qPCR. FIA cards are excellent media to collect, transport and store samples for MD diagnosis and to monitor MD vaccines according to the results of some studies (Shambhu *et al*., 2012).

## 2.5 Differential Diagnosis

Marek's disease, lymphoid leukosis (LL) and reticuloendotheliosis (RE) are three illnesses that have many similarities in appearance. Furthermore, peripheral neuropathy (PN) syndrome is defined by nerve lesions that are virtually indistinguishable, both clinically and histopathologically, from those generated by MDV. It was first described in SPF hens and later documented in commercial flocks. The start of neurological clinical indications around 6 weeks of age, as well as expansion of peripheral nerves in the absence of visceral tumors, are all diagnostic criteria for PN to be consider as a differential diagnosis (Gall *et al*., 2018).

Isolation of the MDV virus, evidence of viral DNA or antigens in tissues and antibody detection are the main approaches for detecting infection. MDV infection in a flock can be detected by isolating the virus from affected chicken tissues. However, because of MDV's widespread nature, the diagnosis of MD should be based on a combination of MDV isolation or genome detection by very sensitive PCR, as well as clinical disease (Schat and Nair, 2008).

**Table 2:** Features useful in differentiating Marek's disease, lymphoid leukosis and reticuloendotheliosis (OIE, 2018).

| | Feature | | | | | | |
|---|---|---|---|---|---|---|---|
| **Disease** | **Age** | **Signs** | **Incidence** | | **Neural involvement** | **Bursa of Fabricius** | **Tumours in skin, muscle and proventriculus, 'gray eye'** |
| **Marek's disease** | Any age. Usually 6 weeks or older | Frequently paralysis | Frequently above 5% in unvaccinated flocks. Rare in vaccinated flocks | **Macroscopic lesions** | Frequent | Diffuse enlargement or atrophy | May be present |
| **Lymphoid leukosis** | Not under 16 weeks | Non-specific | Rarely above 5% | | Absent | Nodular tumours | Usually absent |
| **Reticuloendotheliosis*** | Not under 16 weeks | Non-specific | Rare | | Infrequent | Nodular tumours | Usually absent |

| Feature | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Neural involvement** | **Liver tumours** | **Spleen** | **Bursa of Fabricius** | **Central nervous system** | **Lymphoid proliferation in skin and feather follicles** | **Cytology of tumours** | **Category of neoplastic lymphoid cell** |
| Yes | Often perivascular | Diffuse | Interfollicular tumour and/or atrophy of follicles | Yes | Yes | Pleomorphic lymphoid cells, including lymphoblasts, small, medium and large lymphocytes and reticulum cells. Rarely can be only lymphoblasts | T cell |
| **Microscopic lesions** | | | | | | | |
| No | Focal or diffuse | Often focal | Intrafollicular tumour | No | No | Lymphoblasts | B cell |
| Infrequent | Focal | Focal or diffuse | Intrafollicular tumour | No | No | Lymphoblasts | B cell |

*Reticuloendotheliosis virus may cause several different syndromes. The bursal lymphoma syndrome is most likely to occur in the field and is described here.

## 2.6. **Gross and Histopathological Lesions of Marek's Disease**

Microscopic lesions appear one to two weeks after infection and gross lesions appear three to four weeks later. Enlargement of the peripheral nerves and spinal ganglia, as well as lymphoma development in many organs and tissues, are common gross changes of MD. There are also skin lesions, which are more common in meat-type chickens than in egg-type chickens. Lymphomas can develop in almost any tissue or organ. MD tumours are found in gonads, liver, spleen, muscle, heart, kidneys, proventriculus and intestines. The tumour can be diffuse, nodular, or both in its distribution in a tissue or organ. Viscera seem enlarged, sometimes to several times their usual size and discolored grayish to whitish when dispersed (Fenner *et al*., 2011).

Skin lymphomas are typically seen in the follicles of feathers and are more visible in dressed carcasses. A few or several follicles grow and lesions from multiple follicles merge in severe cases. There are whitish nodules at first; however they can become ulcerated with dark crust forming in some cases. Comb and wattles are also susceptible. One of the most common lesions associated with MD is peripheral nerve enlargement. Gross lesions in the nerve are seen in any or many nerves like sciatic, brachial, vagus and mesenteric nerve. The nerves become rounded instead of normal flat and slightly striated appearance. There are two types of lesions: unilateral and bilateral. Nerves or nerve regions that have been impacted can be up to 2–3 times their normal thickness. The enlarged nerves lose their normal glistening cross-striations, have a gray or yellow discoloration and sometimes an oedematous and haemorrhagic appearance in severe cases (Gimeno, 2014).

Histopathologically, infiltration of lymphocytes is the most characteristic lesion. The lesions are classified as Type I if there is diffuse infiltration of the lymphocytes and cells looking like plasma cells, Type II if nerves show oedema with little lymphocyte infiltration, and Type III if there is infiltration of lymphoblastic cells forming tumorous follicles. MDV-induced lymphomas share the same microscopic features regardless of location. As a result, a general description is given, followed by details about the various tissues. MD tumours can be localized, clearly distinguishing themselves from the normal visceral parenchyma, or they can infiltrate and destroy the normal parenchyma. Small and medium lymphocytes, lymphoblasts and macrophages make up MD lymphomas (Payne, 1985).

Plasma cells are present quite rarely. Pleomorphic lymphoid tumour cells have a large amount of cytoplasm and few organelles outside mitochondria and ribosomes. The nuclei are large and irregularly shaped, with visible nucleoli. Because MD lymphomas have an inflammatory component, it might be difficult to tell the difference between early stages of the neoplasm and normal lymphoreactive areas or severe inflammatory lesions caused by MDV (Gimeno, 2014).

There are two forms of peripheral nerve lesions: type A and type B. Type A is distinguished by a large invasion of neoplastic cells that disrupts the nerve's structure. Lymphomas in nerves have the same microscopic characteristics as lymphomas in other tissues. Type B lesions are inflammatory in nature and are marked by plasma cell infiltration, oedema and varying degrees of demyelination (Lawn and Payne, 1980).

## 2.7. **Distribution of Marek's Disease**

In 1907, Dr. Joseph Marek identified the condition as rooster paralysis (Marek, 1907). MD almost destroyed the chicken industry in the 1960s, but once HVT was discovered to be identical to MDV and live vaccinations were created in the 1970s, the illness was brought under control. Then, the virulence of variant MDV increased. As a result, several MD outbreaks have been documented worldwide and new vaccinations have been created to tackle more virulent MDV. Currently, highly virulent isolates of MDV are circulating worldwide and are capable of outsmarting current vaccine protection. Marek's disease has cost the chicken business a lot of money all around the world (Shambhu *et al*., 2012).

Marek's disease virus is found in all poultry-producing areas around the world. MD outbreaks have recently been observed all throughout the world, even in flocks that have been vaccinated (Okwor and Eze, 2011). Only five nations, including Peru in Latin America, recorded economic losses due to MD since the 1990s, according to a questionnaire study used to analyze the incidence of MD in 55 countries (Gimeno, 2014).

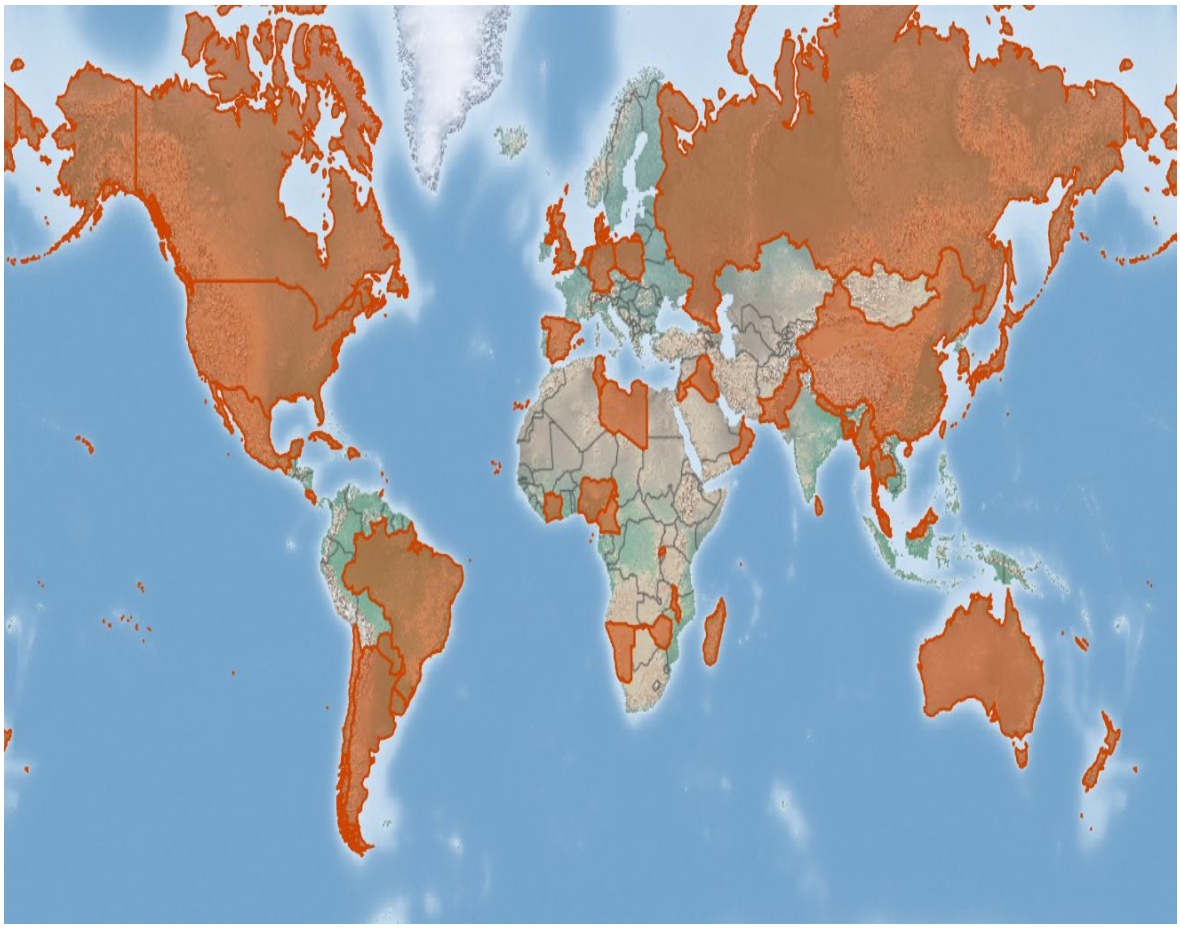


**Figure 3:** World distribution of Marek's disease

**A)** MD confirmed to be present by OIE (reddish-orange color areas) and **B)** No information on the presence of MD (other areas)

**(https://www.cabi.org/isc/datasheet/76376#toDistributionMaps)**

*2.7.1 Status of Marek's disease in Ethiopia*

Marek's disease was initially identified in Ethiopia in 1983, with a reported incidence rate of 0.3% in commercial chicken farms between 1983 and 1986 (Alamargot, 1987). Lobago and Woldemeskel (2004) investigated an MD outbreak in a commercial poultry farm in central Ethiopia with 8500 chicks. For the first 14 weeks of the outbreak, the mortality rate was 46%, highlighting the outbreak's potential importance for current chicken farming in the country.

Marek's disease has become a major problem in Ethiopia's chicken business. According to Duguma *et al*. (2005), the magnitudes of morbidity and mortality on indigenous chickens

in Ethiopia were practically identical, 340/503 and 333/503, showing that MD is highly lethal to the native breeds (97.9% fatality). The research was carried out at the Debrezeit Agricultural Research Center in central Ethiopia using local chickens obtained from various parts of the country. In every case, the disease's presence in small-scale commercial flocks and backyard chicken farms indicates that the disease is widespread in the country, necessitating a thorough prevalence and epidemiological examination (Duguma *et al*., 2005).

Isolation and molecular detection of MDV from outbreak samples were carried out in central Ethiopia, revealing that 28 out of 30 pooled outbreak samples treated with a conventional PCR test were positive for MD (Mirtneh, 2015). Berhan (2014) found that all Marek's disease virus isolates were clustered under Gallid Herpes virus type 2 in a study on the isolation and molecular characterisation of Marek's disease virus from clinically ill chickens produced under diverse production systems in central Ethiopia (Berhan, 2014). In a recent study by Yimer *et al*. (2021), the isolation of the Marek's Disease Virus serotype-1 was confirmed too. The *meq* gene sequence data was presented in the previous study with the result of genetic distance between Bishoftu and Mojo isolates at 0.000 (100% similar, not different). Comparison of the *meq* gene nucleotide sequence of these isolates with that of 20 reference strains showed a high similarity with virulent BC-1 strain of USA (genetic distance of 0.004 or 99.996%) and virulent MPF57 and 04CRE strains of Australia (0.007 and 0.009 genetic distance respectively) (Yimer *et al*., 2021).

### 2.8. **Control and Prevention of Marek's Disease**

Marek's disease management is based on three principles: strong biosecurity, genetic resistance selection and vaccinations. Keeping birds in virus-free premises and filtered air circulation is also important. Biosecurity and genetic resistance, rather than being major control measures, are currently used as key adjuncts to vaccinations (Gimeno, 2014).

The importance of genetic resistance in the control of MD was originally recognized some decades ago and it was the first approach of disease control. MD resistance in chickens is influenced by two types of genes: MHC genes and non-MHC genes. The importance of MHC genes in MD resistance has been extensively established and documented. Many non-MHC genes may also have a role in resistance (Calnek, 2001)

If optimum biosecurity measures are followed, MD can be eradicated from a farm. Unfortunately, the chicken business does not have the financial resources to use filtered air and positive pressure housing. A good biosecurity plan, on the other hand, will greatly aid in the control of MD. Any biosecurity plan should take into account two key factors: Reducing the initial levels of MDV in the farm by preventing MDV from entering the building and preventing MDV from exiting the building to pollute the environment. There are two further issues that need to be addressed: air quality and the disposal of chicken remains and manure. Biofilters designed to eliminate odours from air coming from livestock facilities could be a way to reduce MDV contamination in the air coming from chicken farms. Composting has been proven to be a viable alternative to throwing away both carcass and manure (Spencer and Guan, 2004).

The cornerstone of MD protection is modified live vaccinations. There are three MDV serotypes represented by a significant number of licensed MD vaccines. HVT alone, HVT plus a serotype 2 strain and CVI988 (Rispens) with or without viruses of serotypes 2 and 3 are the three types of formulations in ascending order of efficacy. Because most chicks are exposed to MDV within a few days after being placed in their brooding quarters, early immunization is critical. MD vaccinations are therefore given to chicks at hatch via subcutaneous or intramuscular inoculation, or inovo via amniotic or intraembryonic routes (Witter *et al*., 2005).

## 3. MATERIALS AND METHODS

### 3.1. Study Area

The study took place in poultry farms with Marek's disease outbreaks in Bishoftu town from January 2022 to May 2022. Bishoftu town is the main area for poultry production in Ethiopia. Bishoftu is located in Oromia National Regional State approximately 45 km south east of Addis Ababa and is at an altitude of 1850 meters above sea level. This area experiences a bimodal rainfall pattern with a short rainy season from March to May and a long rainy season from June to October. The area has an average annual rainfall of 800 mm and average maximum and minimum temperatures of $28^0$C and $12.3^0$C, respectively. It is found at $9^0$N latitude and $39^0$E longitude (Gissila, 2001).

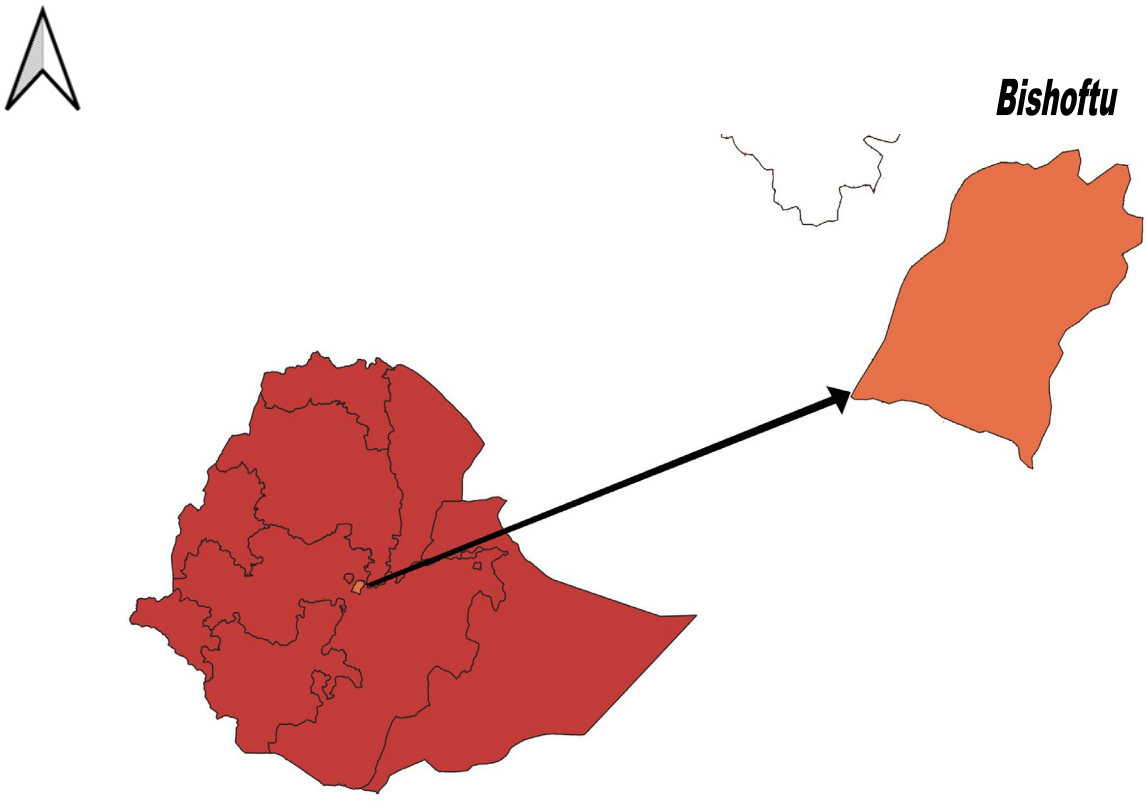



**Figure 4:** Map showing the study area (https://gadm.org/ )

### 3.2. Study Animals

The study was conducted in chickens that were found in farms where outbreaks of Marek's disease occurred. Chickens showing clinical signs of Marek's disease in the susceptible age group reared under three different intensive farms were examined in the study. For the sake of clarification the first, second and third farms were named Farm AK, Farm BD and Farm PA, respectively and will be addressed as such from this point onward. A total of 70 chickens: 30 from Farm AK, 25 from Farm BD and 15 from Farm PA from three different poultry flocks in Bishoftu town were examined. These included one layer farm (Farm AK) that had 2800 chickens and two broiler farms (Farm BD and Farm PA) that had 1820 and 6400 chickens respectively. Five chickens that didn't show clinical signs but were in contact with the sick chickens were also sampled.

### 3.3. Study Design and Technique

The study used a cross sectional study design on three suspected Marek's disease outbreak farms in Bishoftu. Farm AK is an intensive layer farm that had 2800 chickens which produced approximately 2500 eggs/day. Farm BD and Farm PA are intensive broiler farms that had 1820 and 6400 chickens respectively. Both farms use an all-in and all-out management system. All three farms practice good biosecurity measures such as dipping in foot bath before entering the poultry house. The study was conducted through virus isolation and molecular detection of Marek's disease virus as well as gross and histopathological lesion characterization of the disease.

### 3.4. Sampling Method

The purposive sampling method was used during the research period in which sampling was undertaken from chickens showing clinical signs as well as apparently healthy chickens found in farms of the disease outbreak.

### 3.5. Sample Collection and Transportation

Samples were taken from clinically sick and some apparently healthy chickens of three farms with suspected Marek's disease outbreaks. Suspected clinical cases were collected following careful examination of individual cases. For virus isolation and characterization,

feather follicle samples and some spleen were collected aseptically from suspected clinically sick and apparently healthy chickens. The samples were placed in sterile labelled bottles containing Viral Transport Media (VTM) inside an ice box and transported to the Addis Ababa University Microbiology laboratory and stored at -20$^0$C for a few days. Then the samples were transported to the molecular diagnostic laboratory of the Animal Health Institute (AHI). To maintain the viability of the virus, samples for molecular diagnosis were stored in deep freezers at -20$^0$C until further laboratory analysis was carried out (OIE, 2018). The gross lesion examinations were done at the farm premises where the outbreak cases were and the carcass was disposed of off by burial. Tissue samples of the lungs, heart, sciatic nerve, liver, spleen, ovary and kidneys were collected in 10% formalin for histopathological examination and transported to AHI (Annex 1 and Annex 2).

## 3.6. Laboratory Investigation

### *3.6.1. Virus Isolation*

#### *3.6.1.1. Virus isolation from feathers*

Feather follicles collected from 65 chickens from the three farms were collected in a cryovial tube containing VTM. The feathers were processed individually inside a Biosafety cabinet class II. The feather follicles containing blood were cut at the tip at approximately 0.1 cm and placed inside a tube. Feathers from the same outbreak farm were pooled and processed inside Biosafety cabinet level II to increase the chance of virus DNA isolation.

A 1:10 (wt. /vol) suspension of feather tips in SPGA/EDTA (sucrose, phosphate, glutamate and albumin/ethylenediamine tetra-acetic acid) (Annex 3) buffer was homogenized for 3-5 minutes. The suspension was then transferred into sterile tube and centrifuged at 2000 rpm for 10 minutes. Supernatants were harvested and inoculated onto confluent primary chicken fibroblast cells (Annex 4) in maintenance GMEM (Annex 5) supplemented with 2% bovine foetal calf serum and incubated at 37$^0$C. Cultures were observed daily using an inverted microscope for up to 1 week to check for the presence of characteristic CPE for MDV (Wozniakowski *et al*., 2011). Those that had not shown CPE were blindly passaged up to the second and third passage.

*3.6.1.2. Virus isolation from the spleen*

The spleen samples were first cut into small pieces using sterile scissors inside the Biosafety cabinet class II and further ground using a sterile mortar and pestle. A 10% (w/v) suspension of spleen samples was prepared in sterile phosphate buffered saline (PBS) supplemented with penicillin and streptomycin (Annex 6). The suspension was transferred into a sterile tube and centrifuged at 2000 rpm for 10 minutes. Supernatants were harvested and inoculated onto confluent primary chicken fibroblast cells in maintenance GMEM containing 2% bovine foetal calf serum and incubated at $37^0$C. Cultures were observed daily using an inverted microscope for up to 1 week to check for the presence of cytopathic effects (CPEs) characteristic for MDV. Those that had not shown CPE were blindly passaged up to the second and third passage.

*3.6.2. DNA extraction*

DNA extraction was conducted in the molecular biology laboratory of AHI. Extraction of DNA from 65 feather follicle and 5 spleen tissue samples (Annex 7) was carried out using a DNeasy® Blood and Tissue Kit following the manufacturer's instructions (QIAGEN, Germany). For DNA extraction 180 µl buffer ATL and 20 µl proteinase K was added to the tubes containing the samples and mixed using vortex, centrifuged and then incubated at $56^0$ c for 20 hours. After 20 hours, 200 µl buffer Al was added and mixed for 15 seconds and then the mixture was incubated for 10 minutes at $70^0$ c and centrifuged briefly at 8000 rpm.

After the suspension was centrifuged briefly in centrifuge tubes, 200 µl of ethanol was added and mixed for 15 seconds. This mixture was applied to a QIAamp spin column to precipitate. The QIAamp spin column was placed in a clean collection tube and centrifuged for 1 minute at 8000 rpm. The QIAamp spin column was opened carefully and 500 µl AW1 buffer was added and placed in new collection tubes, and then centrifuged for 1 minute at 800 rpm. The QIAamp spin column was opened carefully and 500 µl AW2 buffer was added and placed in a new collection tube and then centrifuged for 3 minutes at 20,000 rpm. The QIAamp spin column was placed in a sterile reaction vessel and 200 µl of AE buffer was added. Then, the column was incubated for 1 minute at room temperature and centrifuged for 1 minute at 8000 rpm and the filtrate was retained at $4^0$C for 48 hours.

Finally, the samples were centrifuged for 1 minute at 35,000 rpm. The eluted DNA yield was used for real-time PCR amplification.

*3.6.3. Real-time Polymerase Chain Reaction*

Real-time PCR offers a faster and more reliable recommended test as a diagnostic tool for Marek's disease (Islam *et al*., 2004). Real-time PCR was performed targeting the amplification of the partial sequence of the *meq* gene. It was performed according to the manufacturer's instructions after DNA extraction from feather follicles and the spleen was done using a QIAamp DNA Mini Kit. The master mix reagent compositions for amplification were as follows: platinum PCR mix, MDV-1 Forward primer (5'-GGAGCCGGA GAG GCTTTA TG-3'), MDV-1 Reverse primer (5'- ATCTGG CCC GAATACAAG GAA-3'), MDV-1 probe (5'-(FAM) CGTCTTACC GAG GAT CCC GAA CAG G (BHQ-1)-3'), Bovine Serum Albumin (BSA), and DNAse and RNAse free $H_2O$ water mix. All reagents were mixed in a single reaction tube (master mix) to ensure the reagents were distributed homogeneously. After the mixture was ready, 15 µl of the master mix (Annex 8) was added to 5 µl of each sample onto the applied biosystem plate.

Finally, real-time PCR was carried out in a final reaction volume of 20 µl using a 200 µl capacity thin wall PCR tube. As the positive and negative controls, known positive MDV antigen and DNAse and RNAse free water were used as positive and negative controls, respectively. The plates were sealed with a sealer to avoid evaporation and placed in the thermal cycler machine that was connected to the computer with its own software (Applied Biosystems Sequence Detection Software), and the program was adjusted accordingly for each specific reaction. The PCR protocol was performed with an initial denaturation at $95^0$C for 15 minutes, followed by 45 cycles of amplification, denaturation at $95^0$C for 15 seconds, annealing at $60^0$C for 15 seconds and extension at $72^0$C for 10 seconds (Annex 9). Samples with a cycle threshold value (CT value) of greater than 36 were considered negative based on the positive control's CT value.

*3.6.4. Histopathological examination*

Different tissue samples were collected and submitted to the pathology laboratory of AHI that was stored and preserved in 10% formalin. Part of the tissue that had gross visible lesions was trimmed in such a way that the lesion were included or not missed and to fit standard histological processing tissue cassettes (5 mm thickness). The tissues then underwent tissue specimen processing that consisted of fixation of tissue by formalin, dehydrating tissue by increasing alcohol concentrations, clearing of tissue by xylene and impregnation of tissue by paraffin wax. Impregnated tissue was placed in a mould with labels and then fresh melted wax (54-60$^0$C) was poured and allowed to settle and solidify. Tissue sections with a thickness of 4-5 microns were placed in a water bath to straighten the ribbon and then adhered to the surface of frost end and clear slide and stained with haematoxylin-eosin dye. The stained samples were examined under a light microscope for histological changes (Votano, 2007).

## 3.7. Data Analysis

The data collected during sampling and laboratory analysis were coded and stored in a Microsoft office Excel spread sheet 2010. The data was analyzed using simple descriptive statistics.

## 3.8. Ethical Statement

All procedures were approved by the ethics committee of Addis Ababa University College of Veterinary Medicine (Ref. No: VM/ERC/05/02/14/2022).

## 4. RESULTS

### 4.1. Clinical Examination, Disease Occurrence and Gross Lesion Findings

A total of 70 chickens: 30 from Farm AK, 25 from Farm BD and 15 from Farm PA from three different poultry flocks in Bishoftu town were examined. These included one layer farm (Farm AK) that had 2800 chickens and two broiler farms (Farm BD and Farm PA) that had 1820 and 6400 chickens respectively. Representative spleen and feather samples were collected from 40 female and 10 male chickens for detection of the virus (Table 3). The disease appeared at 4 months, 3 weeks, and 4 weeks of age in Farm AK, Farm BD, and Farm PA respectively and it killed approximately 300 (10.71%), 358 (19.6%) and 400 (6.25%) chickens, respectively. All three farms practiced good biosecurity measures, gave vaccinations of important diseases on time but as for the history of vaccination against Marek's disease is not known in the case of Farm AK but both Farm BD's and Farm PA's chickens were not vaccinated against MD.

**Table 3: Description of chickens examined for Marek's Disease**

| Town | Date of collection | Type of Sample | | Age group | Farming system |
|---|---|---|---|---|---|
| | | Feather | Spleen | | |
| Bishoftu (Farm AK) | 21/02/2022 | 28 | 2 | 4 months | Intensive (Layer) |
| Bishoftu (Farm BD) | 17/03/2022 | 23 | 2 | 27 days | Intensive (Broiler) |
| Bishoftu (Farm PA) | 18/03/2022 | 14 | 1 | 37 days | Intensive (Broiler) |
| **Total** | | 65 | 5 | | 70 |

Upon clinical examination, the diseased birds showed clinical signs of paralysis (leg and wing), transient paralysis in which affected chickens showed flaccid neck paralysis suddenly, weight loss, 'gray eye', depression, laboured breathing and nasal discharge, raised and roughened skin around feather follicles and a sharp decrease in egg production on the poultry farm (Figure 5).

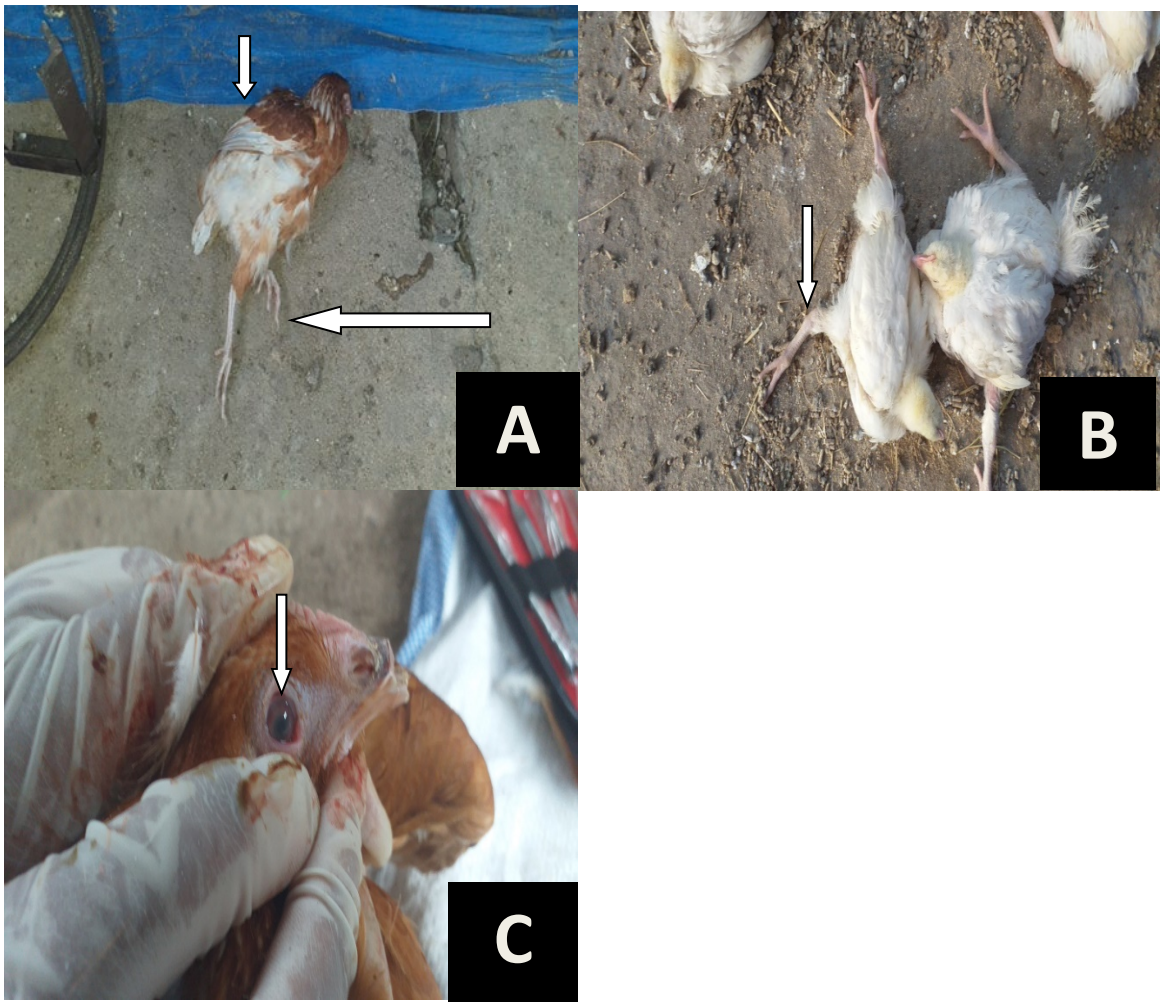
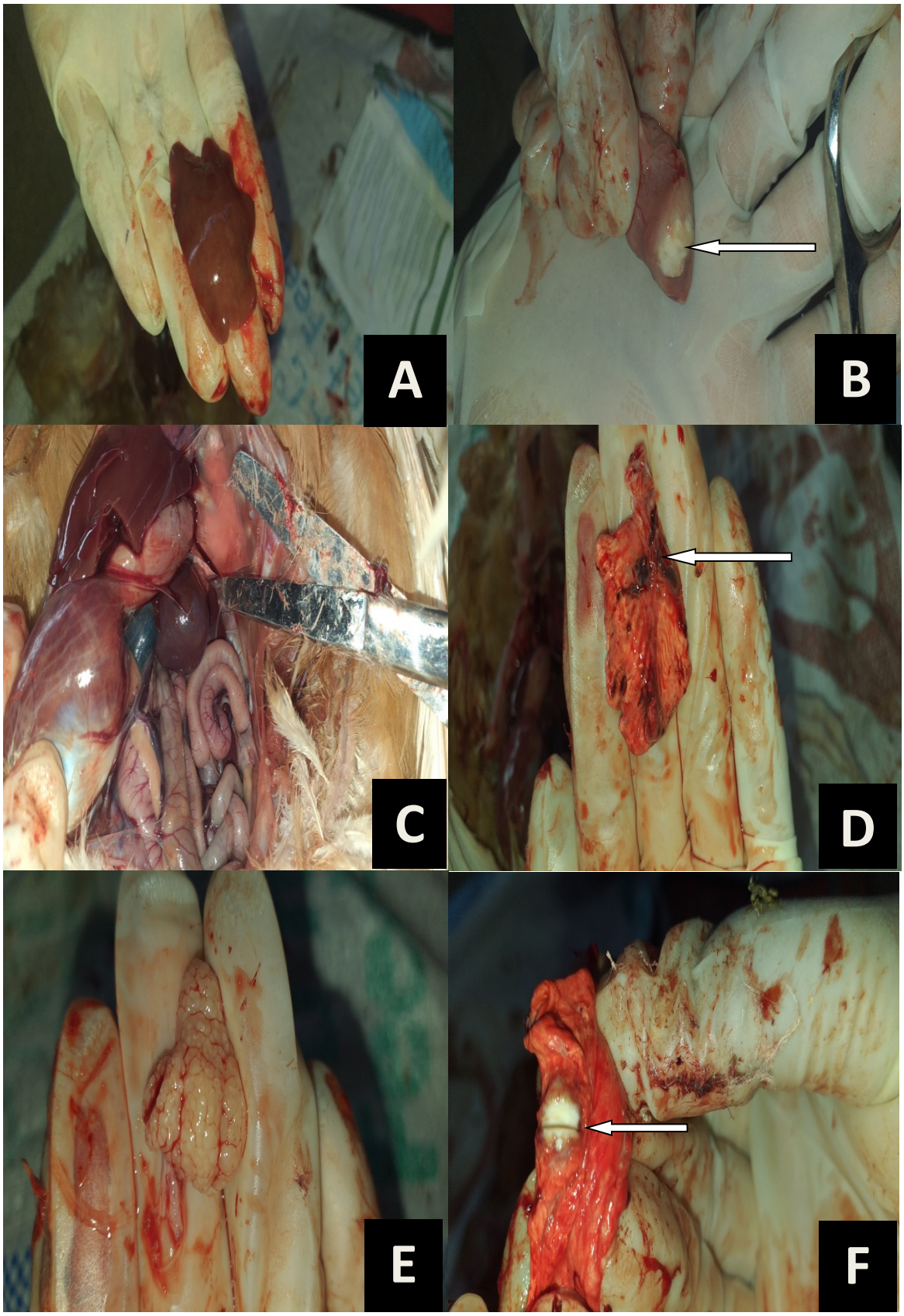



**Figure 5:** Clinical signs of Marek's diseases observed

A) Leg paralysis B) Wing and leg paralysis and C) 'Gray eye'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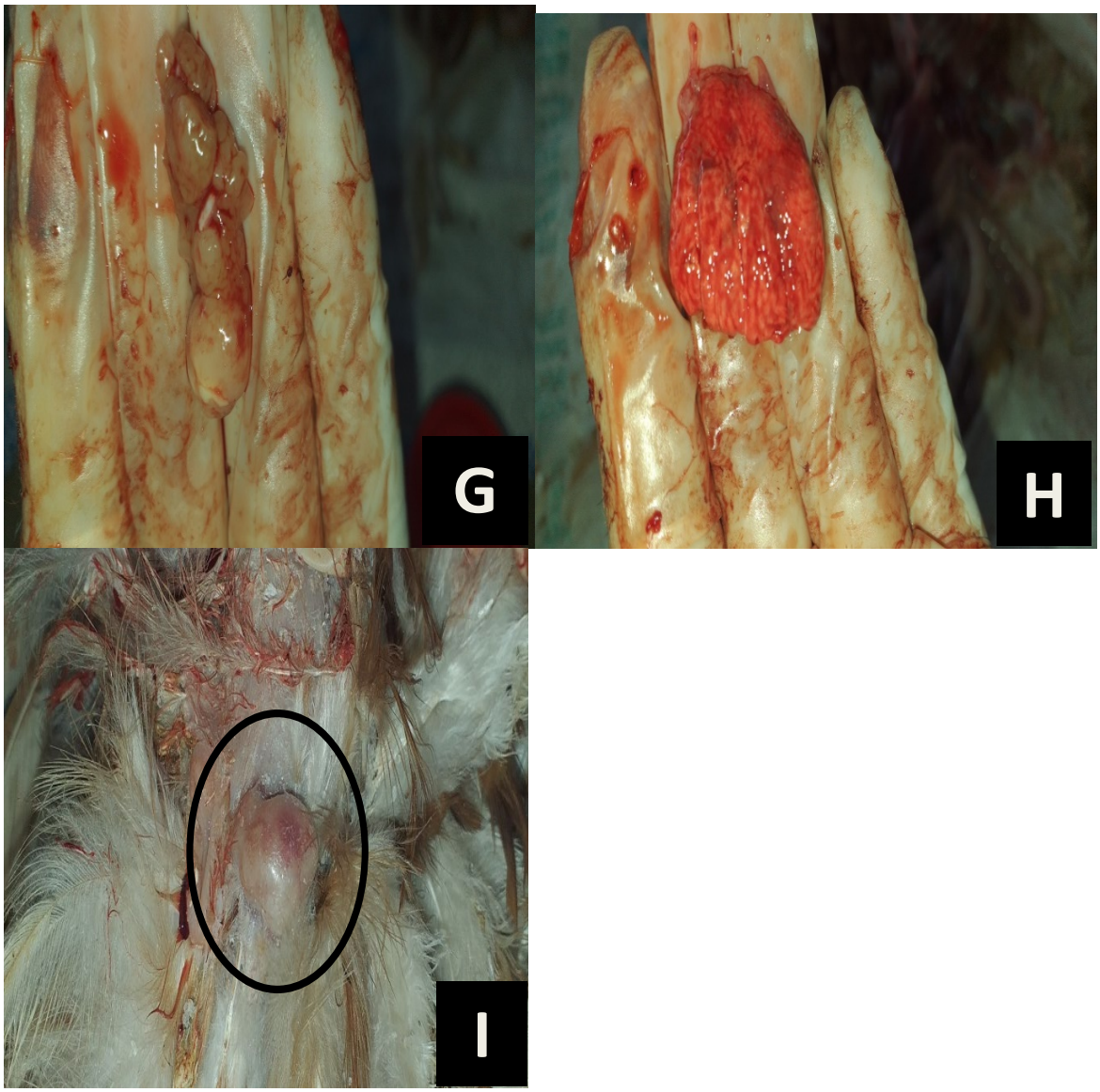



**Figure 6:** Gross lesions observed in affected chickens.
Hepatomegaly (A), Nodule in the heart (B), Splenomegaly (C), Haemorrhage in lung (D), Typical cauliflower appearance of the ovary (E), Nodule in the lung (F), Nodules in the ovary (G) Emphysematous lung (H) and Nodule on the skin (black circle) (I).

Upon gross pathology examination, the following observations were made in different organs that were examined.

*Skin and eyes:* Two (2.85%) of the 70 examined chickens showed nodules on the skin and gray discoloration of the eyes (Table 4).

*Sciatic nerve:* None of the examined chickens showed nerve enlargement.

*Bursa of Fabricius*: Atrophy of the Bursa was seen in most of the younger flocks, some also had yellowish discoloration and yellowish colored fluid in the Bursa of Fabricius (Table 4).

*Liver:* The affected livers were enlarged with rounded edges (hepatomegaly) with diffused petechial haemorrhage in some livers. Some examined livers also showed pale discoloration (Table 4).

*Spleen:* The affected spleens were enlarged with rounded edges and had firm consistency. In some cases, marbled spleen was also observed (Table 4).

*Ovary*: In most cases the ovaries were of normal appearance with no apparent change, but the affected ovaries showed atrophy, cauliflower appearance, and had whitish-yellow nodules of different sizes (Table 4).

*Kidneys:* Most of the kidneys were normal in size and consistency, but in some cases, kidneys had whitish-yellow nodules, atrophied kidneys and fatty consistency (Table 4).

*Heart:* Some affected hearts showed numerous whitish-yellow nodules of different sizes and some showed a single large nodule (Table 4).

*Lung:* Most of the lung lesions found in the layer farm was numerous black discolorations. Although the discoloration is not the result of MD, it might be the reason why some chickens showed respiratory problems such as laboured breathing and nasal discharge. The affected lungs also showed marbled appearance, enlargement and rounded edges (emphysema), haemorrhagic appearance and had whitish-yellow numerous nodules of different sizes (Table 4).

*Other organs:* The oral cavity, trachea, oesophagus, proventriculus, crop, gizzard, both small and large intestines were of normal size and shape and did not show any apparent change.

**Table 4:** Gross lesions seen in different organs in chickens infected with Marek's disease virus

| Organs examined | Gross lesions | No. of chickens showing the lesions | Percentage of chickens showing the lesions |
|---|---|---|---|
| **Eyes** | Gray discoloration | 2 | 2.85% |
| **Bursa of Fabricius** | Atrophy | 44 | 62.8% |
| **Liver** | Hepatomegaly | 38 | 54.28% |
| | Petechial haemorrhage | 9 | 12.85% |
| | Pale liver | 5 | 7.14% |
| **Spleen** | Splenomegaly | 16 | 22.85% |
| | Marbled spleen | 3 | 4.2% |
| **Ovary** | Multiple nodules | 2 | 2.85% |
| | Atrophy | 1 | 1.42% |
| | Cauliflower appearance | 2 | 2.85% |
| **Kidney** | Pale kidney | 5 | 7.14% |
| | Numerous white nodules | 3 | 4.28% |
| | Atrophy | 2 | 2.85% |
| **Heart** | Numerous whitish-yellow nodules | 7 | 10% |
| **Lung** | Marbled lung | 3 | 4.28% |
| | Emphysematous lung | 16 | 22.85% |
| | Numerous nodules | 3 | 4.28% |
| | Haemorrhagic lung | 30 | 42.85% |
| **Skin** | Skin nodules | 2 | 2.85% |

The sum of chickens showing gross lesions indicative of Marek's disease is shown below for the three farms separately (Figure 7, 8 and 9).Visceral nodules were observed in Farm AK (layer farm) but not in Farm BD and PA (broiler farms).

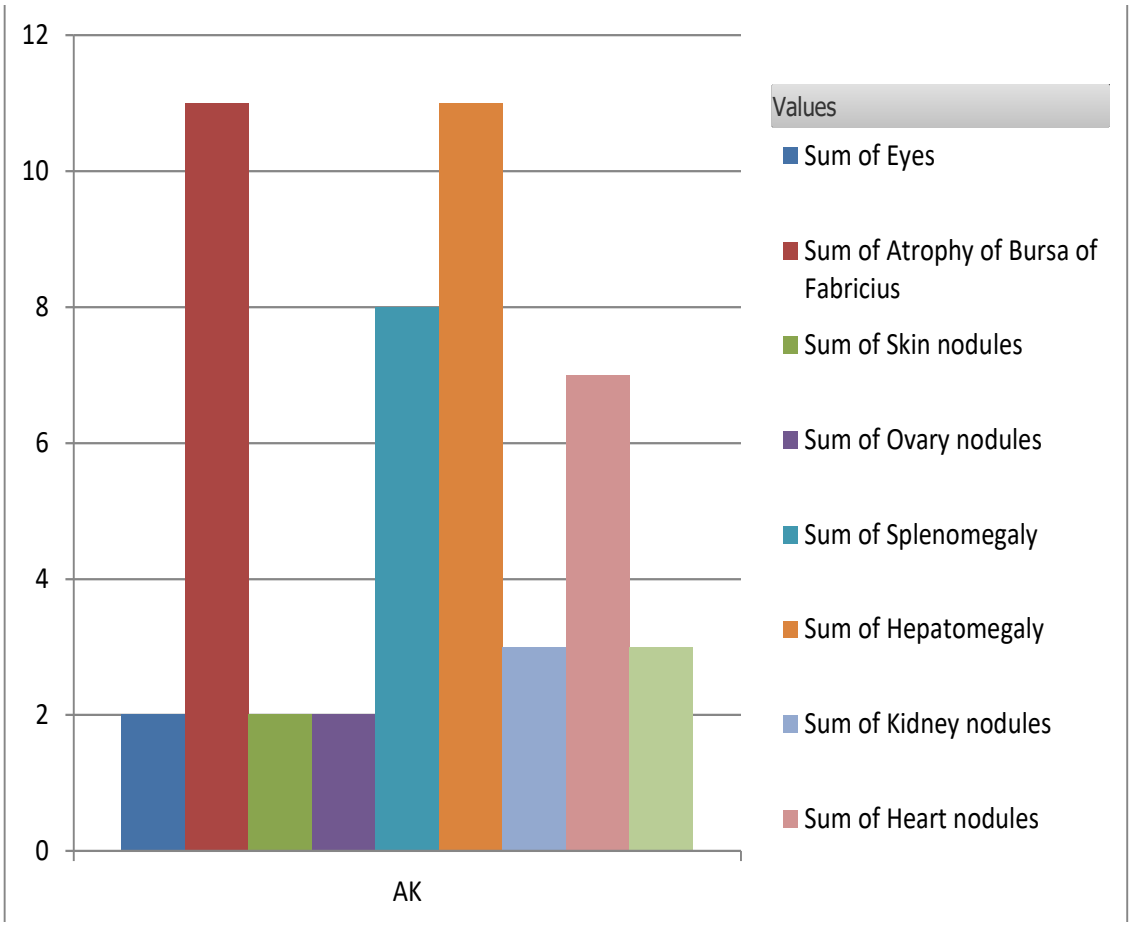



**Figure 7:** Bar chart of the total number of chickens that showed the main gross lesions indicative of Marek's disease found in Farm AK

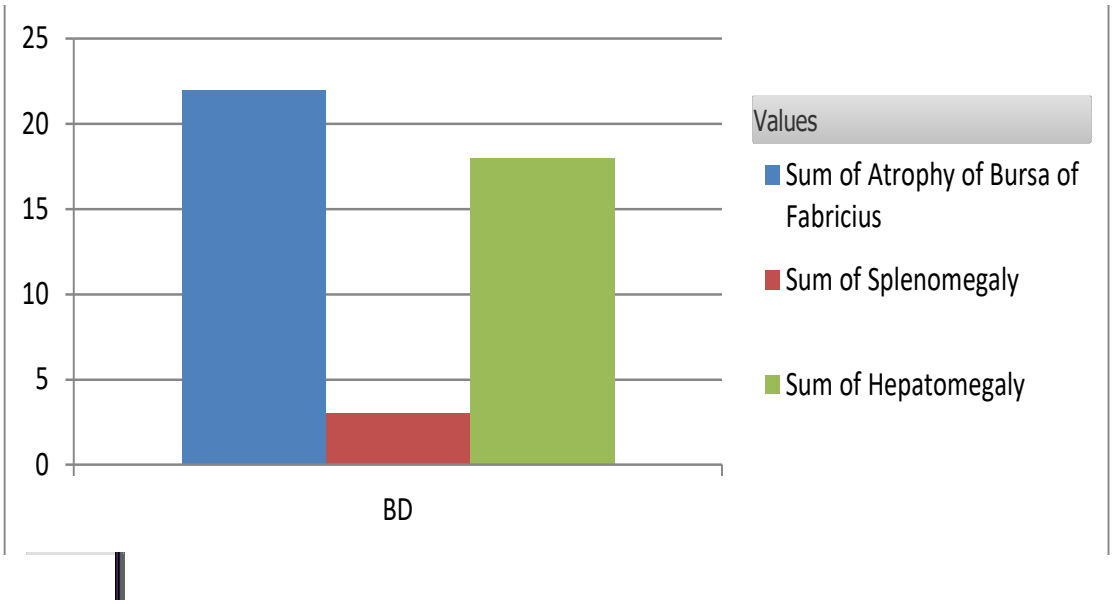



**Figure 8:** Bar chart of the total number of chickens that showed the main gross lesions found in Farm BD


**Figure 9:** Bar chart of the total number of chickens that showed the main gross lesions found in Farm PA

When we compare the lesions seen in the three farms, chickens in Farm BD and Farm PA showed Atrophy of the Bursa of Fabricius, splenomegaly and hepatomegaly frequently than chickens in Farm AK. The average of individual lesions observed in the three farms is summarized in the figure below (Figure 10).

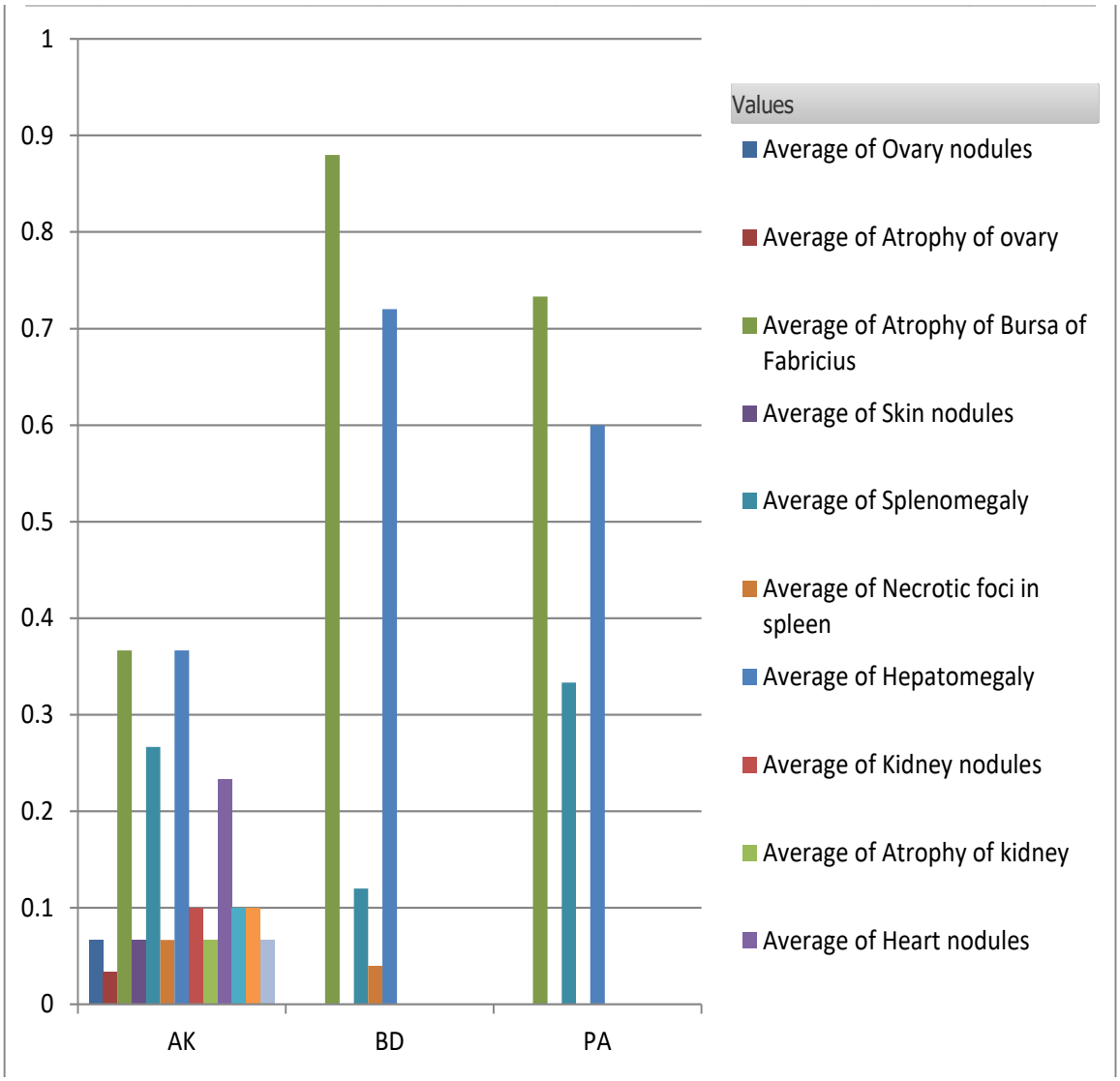



**Figure 10**: Bar chart comparison of the average of important gross lesions seen in the three farms

### 4.2. Detection of the Viral DNA by Real-time Polymerase Chain Reaction

Real-time PCR was performed targeting to amplify a fragment of the *meq* gene of MDV using the MDV-1 forward primer (5‘- GGAGCCGGA GAG GCTTTA TG-3’) and MDV-1 reverse primer (5‘- ATCTGG CCC GAATACAAG GAA-3‘) that are specific to the wild strain of the MDV serotype 1 virus. This step allowed us to reach a definitive diagnosis of the causative agent of the outbreak. Out of the 70 samples that were tested, 53 (75.71%) were positive and 17 (24.29%) samples were negative (Table 5).

**Table 5:** Result of Marek’s disease virus tested by real-time PCR

| Farm | Sample type | Positive | Negative | |
|---|---|---|---|---|
| **Farm AK** | Feather | 25 | 3 | |
| | Spleen | - | 2 | |
| **Farm BD** | Feather | 20 | 3 | |
| | Spleen | - | 2 | |
| **Farm PA** | Feather | 8 | 6 | |
| | Spleen | - | 1 | |
| **Total** | | 53 | 17 | 70 |

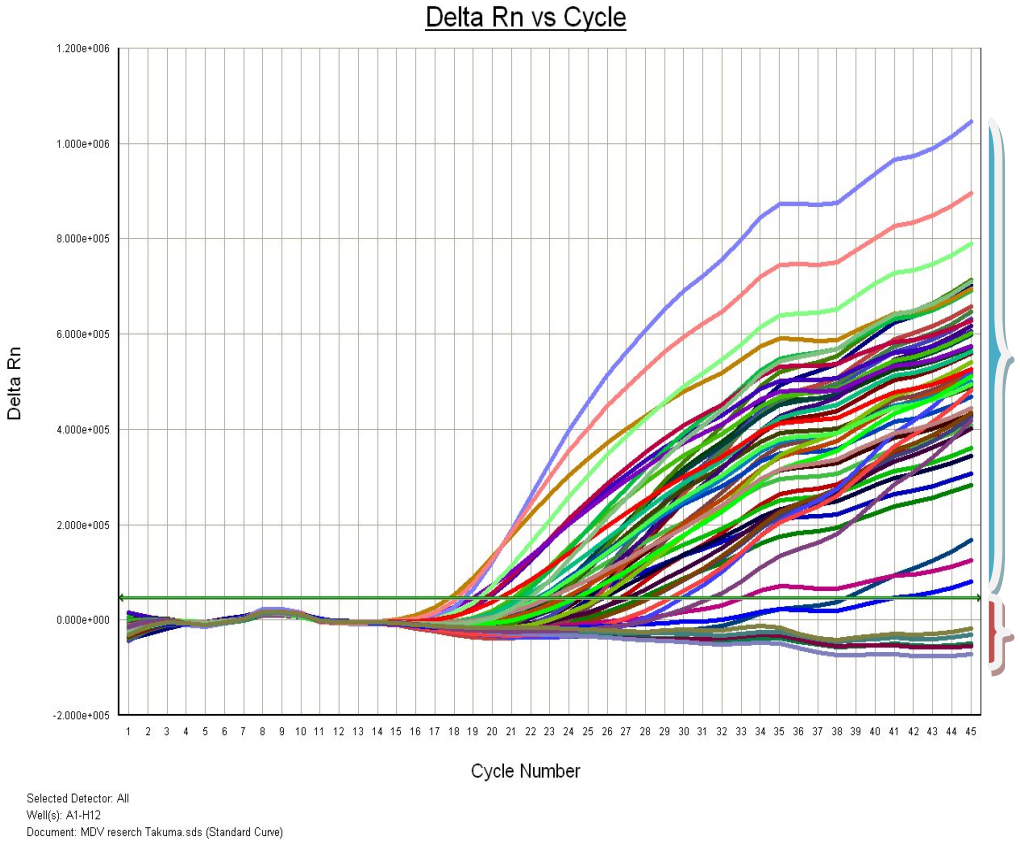



**Figure 11:** Real-time PCR result (standard curve)

Positive samples (blue bracket) and negative samples (red bracket)

### 4.3. Cell Culture and Marek's Disease Virus Isolation

A total of 70 samples, 65 feather follicles and 5 spleens were collected from three flocks of a poultry farm in Bishoftu town. Out of the 53 positive samples, 7 pooled feather samples were isolated and cultured onto chicken embryo fibroblasts (Annex 10). Of the 7 pooled samples, 5 (72.42%) of them showed cytopathic changes. These cells formed foci and rounding of cells that later detached from the wall of cell culture flask causing formation of plaque (Figure 12).

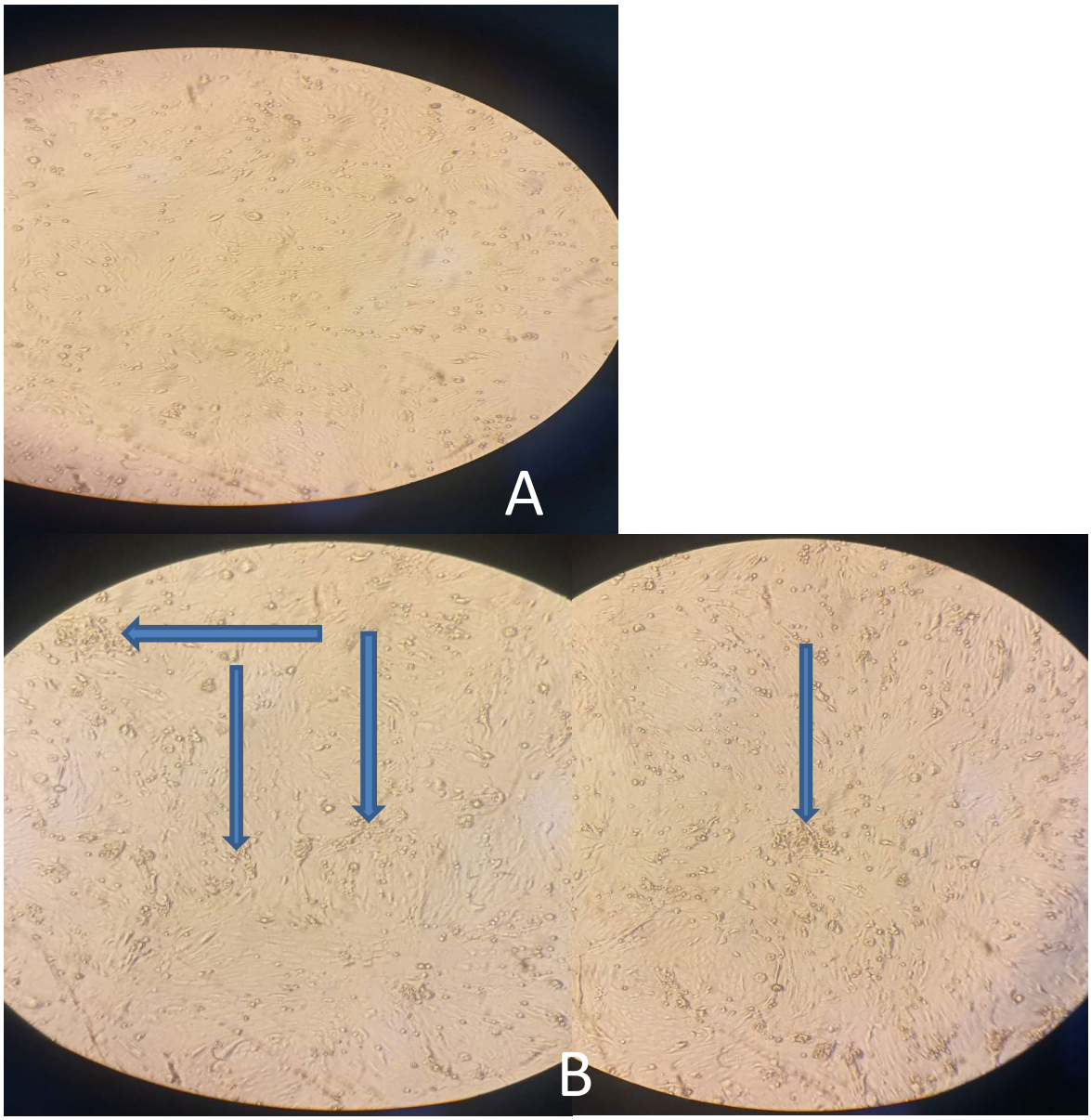



**Figure 12:** Cell culture on chicken embryo fibroblasts.
A) Negative control cell culture and B) characteristic cytopathic effect of MDV (blue arrow)

### 4.4. Histopathological Findings

From the collected tissue samples 17 tissue samples from the sciatic nerve, liver, spleen, heart, lungs and ovary that had specific gross lesions and were PCR positive were chosen for histopathological examination. Tissues were then sectioned, mounted on frosted glass slides, stained with haematoxylin and eosin dye and examined under light microscope (Annex 11).

The characteristic histopathological lesions comprised of infiltration of a large number of lymphocytes, lymphocytic nodules and in some tissues well demarcated pockets of neoplastic lymphocytic nodules. Most of the lesions were not well demarcated and infiltrative type. The detailed microscopic lesions observed in various organs are indicated in photographs as follows:

*Nerve:* Diffuse lymphocytic infiltration of the sciatic nerve.

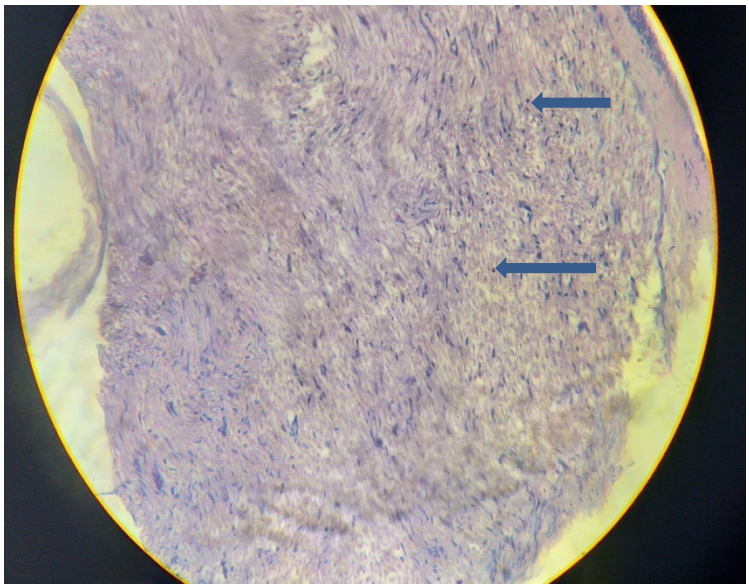


**Figure 13**: Diffuse lymphocytic proliferation in the nerve parenchyma

*Liver*: Multifocal lymphocytic inflammatory nodules with uniform lymphocyte aggregates. These lesions were found, especially around the central veins (A). There was also an observation of lymphocytic nodules with pleomorphic cells. Congestion with a large number of RBCs in dilated veins and dilated capillaries filled with a large number of RBCs (B) and lymphocytic nodule with pleomorphic cells (black circle) (C).

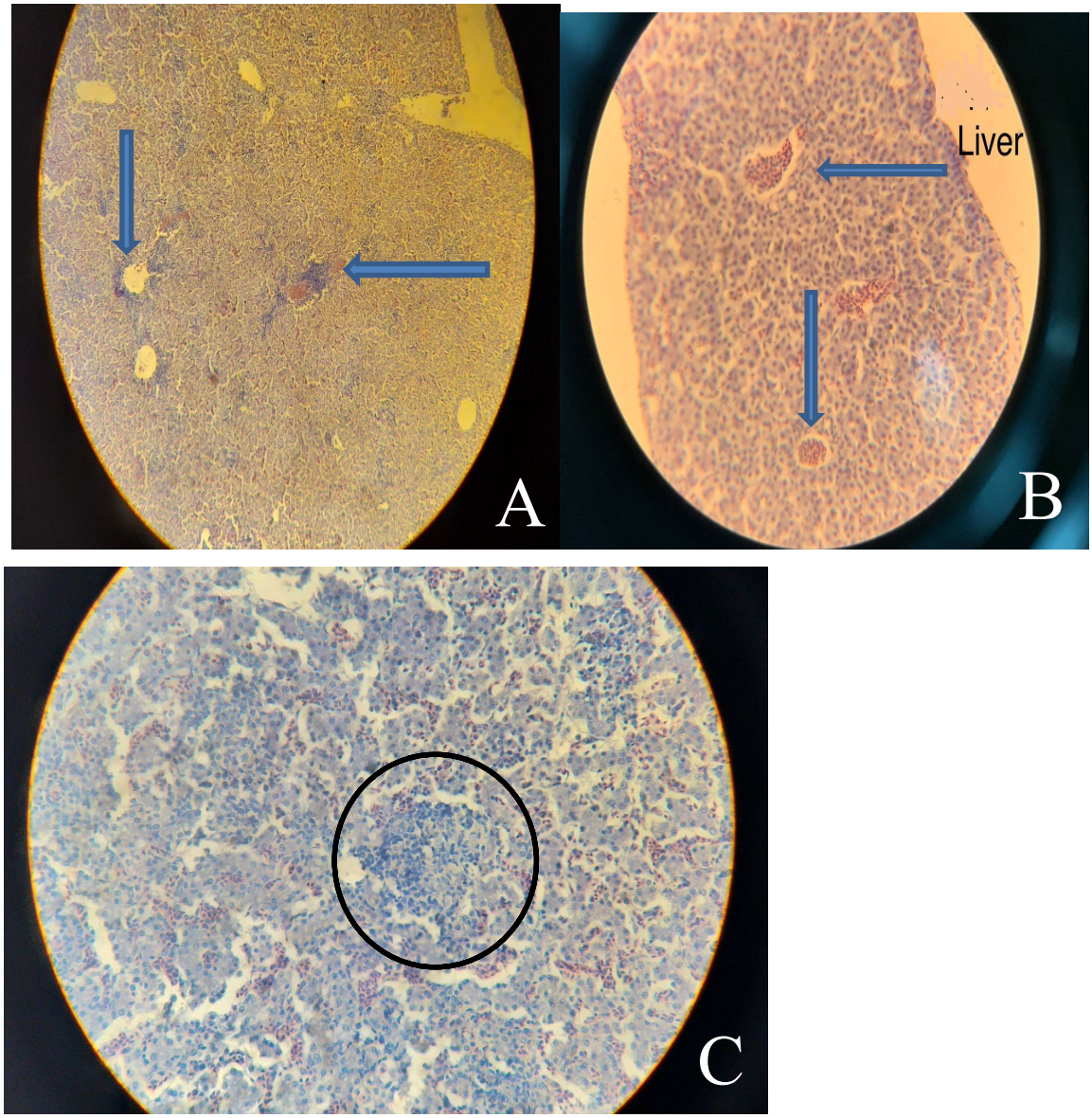



**Figure 14:** Liver histopathology

*Spleen*: Few lymphocytic inflammatory nodules with lymphocyte aggregates and area of lymphocytic depletion and necrosis in the white pulp area.

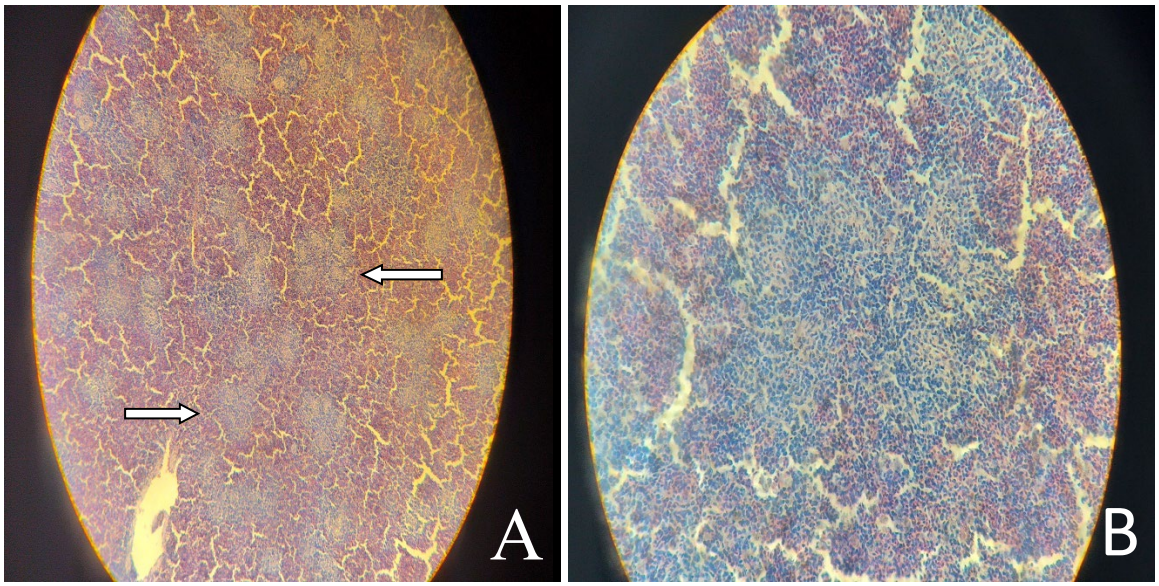
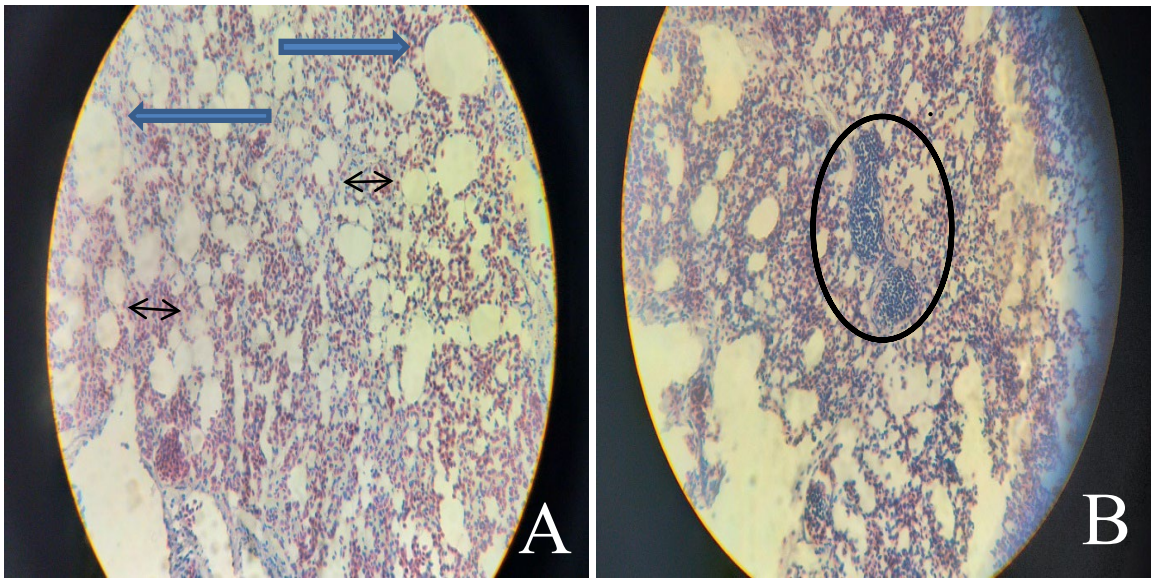



**Figure 15:** Spleen Histopathology

Lymphocytic depletion and necrosis of the spleen (A) and lymphocytic nodules with lymphocyte aggregates (B).

*Lung*: Emphysema (A), interstitial pneumonia (A), asymmetrical pockets of lymphocytic nodules (B) large haemorrhagic areas with free RBCs in the lung mass (C), severe lymphocytic infiltration, and multifocal lymphocytic inflammatory nodules.



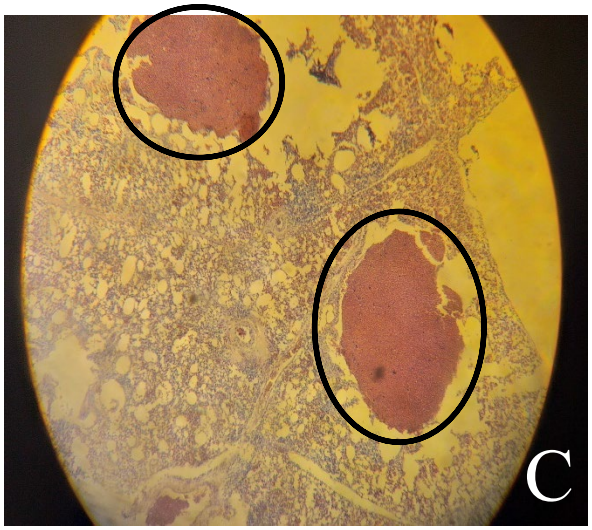
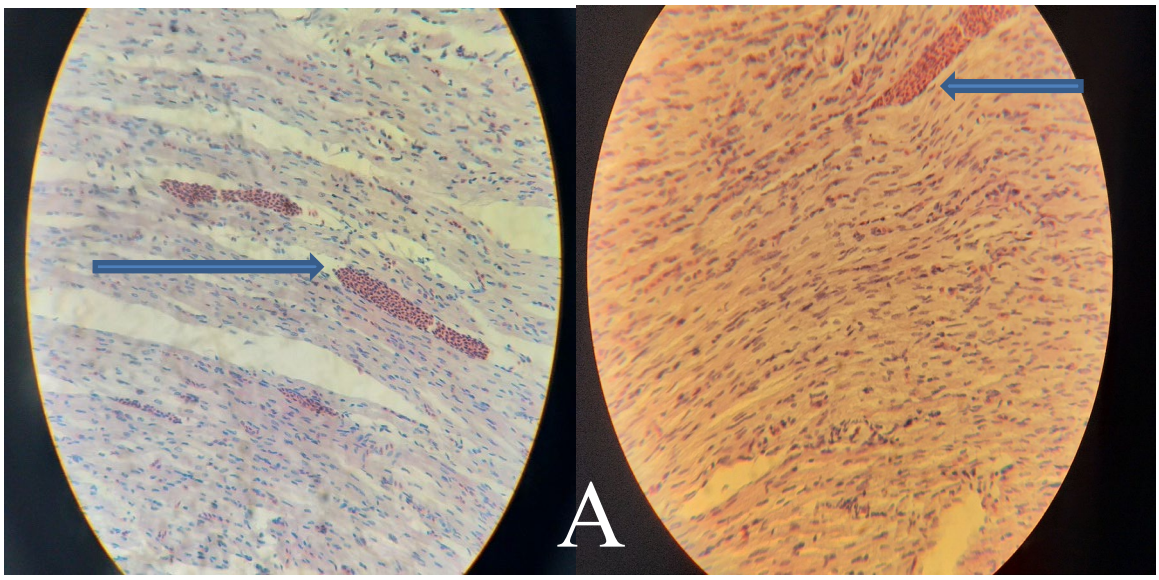



**Figure 16**: Lung Histopathology

*Heart muscle:* few lymphocytic inflammatory nodules with a uniform lymphocyte mass, congestion with large number of RBCs in dilated veins, and capillaries dilated and filled with RBCs (A) and perivascular fibrosis (B) (black circle).



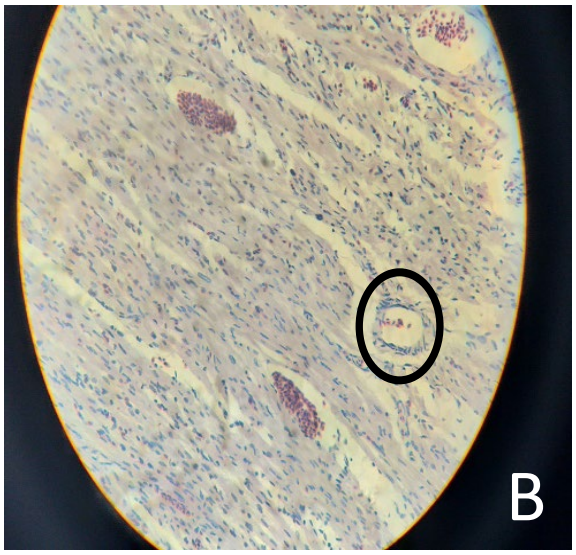



**Figure 17:** Histopathology of heart muscle

Lymphocytic nodules were observed in the lung, liver, spleen and heart. The summary of the sum of the lymphocytic nodules seen during histophatological examination is indicated below (Figure 18).

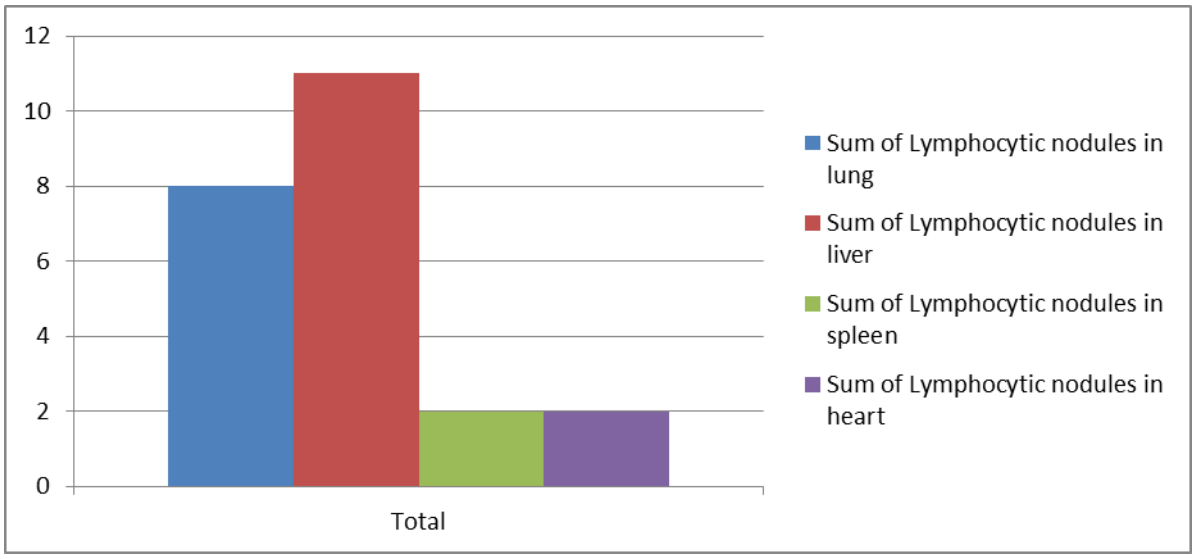



**Figure 18:** Bar chart of the sum of lymphocytic nodules

## 5. DISCUSSION

Marek's disease is a lymphomatous and neuropathic disease of domestic chicken caused by an alphaherpesvirus of the genus Mardivirus called Marek's disease virus. It is one of the most economically damaging infectious illnesses of poultry, with oncogenic T cells infiltrating lymphoid tissues, peripheral nerves, and visceral organs, resulting in a complex pathophysiology that usually results in the death of the infected birds. Infections with the Marek's disease virus have previously been observed in commercial poultry farms in Ethiopia, where it has caused damage to poultry health and output (Duguma *et al*. 2005).

In the present study, Marek's disease virus was isolated and grossly and histopathologically characterized from clinically diseased chickens reared under intensive production systems in three separate poultry farms found in Bishoftu town. In this study, MD outbreak investigation of clinical signs such as depression, loss of weight, raised and roughened skin around feather follicles, 'gray eye', flaccid neck, paralysis of wing, leg and neck and loss of production was observed. According to Marek (1907), the classical form of MD seen in elder chickens is known as 'fowl paralysis'. It involves leg and wing paralysis often with a typical appearance of one leg stretched forward and another leg backward but this was not observed in the older flock (Farm AK) but it was evident in the two younger flocks. In addition, the toes appeared curling and the legs were unable to carry body weight. The flaccid paralysis of neck seen could be due to lymphocytic infiltration of peripheral nerves, spinal cord and associated ganglia with myelin degeneration as Marek (1907) suggests. The clinical signs of MD appeared in most of the studied chickens from as young as 3 weeks old. These clinical signs have also been documented as characteristic signs of MD by OIE report (OIE, 2018). Identification of MDV was confirmed by using the recommended diagnostic techniques of cell culture, and polymerase chain reaction by OIE (2018).

In the classical form of the disease, the nerves are mainly affected, and the mortality rate within a flock rarely exceeds 10–15% (OIE, 2018). In this study, the mortality rate was less than 11% in the two farms but the mortality rate exceeded 15% (19. 6%) only in the case of Farm BD. This may be because the affected chickens were young and their immunity was not that much developed and/or due to secondary infection that was not confirmed in

this study. The mortality persisted for more than 1 month, increasing initially when the outbreak first settled on the farm and then after, it began decreasing. Likewise, the research done by Lobago and Woldemeskel, (2004), observed an increase followed by a decrease in mortality.

The age at which MD outbreaks of occur in chickens is highly variable. During the current study, MD outbreaks were observed in 4-months-old layer chickens, 35-days old and 27-days old broiler chickens. Gross lesions in the visceral organs such as the heart, lung, kidney, and ovary which are indicative of lymphoid tumours were observed in 7(10%), 3(4.28%), 3(4.28%), and 2 (2.85%) chickens that were necropsied, respectively. Based on the calculated average of lesions seen in the three farms, it is evident that mostly the young flocks had lesions in the visceral organs as compared to the adult chickens which showed that the disease dominates in young chickens. The spleen was enlarged and in some cases marbled which is in agreement with Witter *et al*., (1971). Moreover, in the present study nodular masses were observed in the heart, lung, ovary and kidneys. The nodular gross lesions in the visceral organs had firm consistency, smooth surfaces, were whitish-yellow in color and had varying sizes. These post mortem findings are in conformity with Fenner *et al.* (2011). Although, gross pathological changes in the peripheral nerves are common findings in MD as suggested by Calnek and Witter (1991), in the current study, there were no gross changes/ enlargement observed in the peripheral nerves (sciatic nerve) neither in the adult nor young flocks and the occurrence of acute MD without involvement of peripheral nerves has been demonstrated by Fugimoto *et al.* (1971) and Kamaldeep *et al*. (2007).

Most of the diseased birds in this study were of young age and had an atrophied Bursa of Fabricius and this is an important factor in differentiating from lymphoid leukosis, as the latter affects birds older than 16 weeks of age and invariably involves the Bursa of Fabricius (OIE, 2018). Eye lesions have been used as criteria to diagnose MD because retrovirus does not affect eyes. Gross ocular changes, including the loss of pigmentation in the iris (‘gray eye’) was observed in two chickens and is thought to be caused by mononuclear infiltration of the iris. Conjunctivitis was also seen occasionally as stated by Ficken *et al*. (1991). Skin nodules were observed in two chickens in the layer farm but not in the broiler farm, which is in disagreement with the report that says skin nodules are more common in meat-type than in egg-type chickens by Gimeno (2014). The presence of

transient paralysis in the chickens in which they suddenly develop flaccid neck paralysis that become generalized is a supportive diagnosis for the absence of vaccination in the broiler farms. As Gimeno (2014) states TP appears only in unvaccinated chickens that lack maternal antibodies against MDV and in field cases, it has only been diagnosed in unvaccinated flocks of broilers at 3–4 weeks of age, when maternal antibodies have disappeared.

The histopathological findings of the infiltration and proliferation of lymphocytes in different organs, such as the spleen, liver, heart, and lung, suggested MD field infection and were in agreement with the findings of other previous studies (Lobago and Woldemeskel, 2004; Nabinejad, 2013). Most spleen tissues examined under the microscope had lymphocytic depletion and necrosis which coincides with the findings of Hameed and Jihad (2019). The tumour lesions found in the liver were mostly around the central veins and other blood vessels which are in agreement with OIE (2018) which states that liver tumour lesions are mostly perivascular (Table 2).

According to Gimeno (2014), MD tumours can be focal, well demarcated from the normal parenchyma of the viscera, or can infiltrate and destroy the normal parenchyma consisting of a mixture of small and medium lymphocytes, lymphoblast, and macrophages. In this study, multifocal lymphocytic nodules as well huge infiltration by lymphocytes, a mixture of lymphocytes and some macrophages and pockets of well demarcated lymphocytic nodule has been observed. However, only the parenchyma of the lung tissue was observed to be destroyed in this study and no lymphoblasts were seen in the lymphocytic nodules too. An important feature in differentiating MD from lymphoid leukosis is that lymphoid leukosis lymphomatous infiltrations are composed of uniform lymphoblasts while that of MD is composed of heterogeneous population of lymphoid cells. In lymphoid leukosis, gross lymphomas occur in the bursa of Fabricius but in the case of MD the tumour is less apparent (Biswas *et al.*, 2018).

In the RT-PCR assays, the primer pairs specific to MDV-1 serotypes were utilized in a single reaction using DNA extracted from each feather follicles. In accordance, MDV-1 DNA was amplified and this suggests active replication of serotype-1 DNA in the feather follicle epithelium and the circulation of the MDV-1 serotype in the chicken flocks found in Bishoftu town. This calls for strict review of the vaccination strategy of the chick

suppliers/distributors found in the town as well as the strict control of their vaccine practice against MD in day old chicks.

Marek's disease virus isolation on primary chicken embryo fibroblasts was demonstrated on 7 pooled feather samples from the three farm samples. All the isolates were inoculated on CEF and revealed characteristic MDV-induced cytopathic effects of rounding of cells and formation of small plaques with visible small foci of plaques starting from the 5$^{th}$ day of the 2$^{nd}$ and 3$^{rd}$ blind passage and this is in agreement with Demeke *et al*. (2017), Dunn *et al.* (2014) and Heidari *et al.* (2016) who stated that MDV was isolated using chicken embryo fibroblast primary cell culture from most samples collected from clinically diseased chickens. In addition, López-Osorio *et al*. (2017) found the same CPEs of rounded cells and formation of plaques which is characteristic of Herpesvirus.

## 6. CONCLUSION AND RECOMMENDATIONS

In conclusion, Marek's disease is becoming a serious health problem of poultry farms in Ethiopia. This represents a real threat to both local and exotic breeds in backyard and commercial farming systems. The present study confirmed that MDV is circulating in Bishoftu poultry farms based on the detection of the virus using real-time PCR, gross lesion examination results, histopathological evidence such as the presence of lymphocytic lesions in different visceral organs and the characteristic CPE development of plaques and round cells on CEF cell culture of the MDV isolates. MDV is affecting many poultry flocks and causing financial losses to the poultry industry. The currently available MD control options are use of vaccines, selection of resistant stocks, and practicing good biosecurity measures. All these control strategies should be integrated together for a sound control strategy.

Therefore, based on the findings of the present study the following points are recommended:

- ✓ Poultry farm owners, professionals, and the veterinary service of the country should give more attention on the prevention and control of Marek's disease.
- ✓ Full genome sequencing of the present isolates is important to reach a decision on the selection of safe and protective vaccine strain.
- ✓ Further study on the histopathological and gross lesions isolation and molecular characterization of MDV targeting different breeds, age group, and geographical areas on backyard and commercial poultry farms in Ethiopia.

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## 8. APPENDIX

### Annex 1: Tissue preparation and processing for histopathological slide preparation procedures (Votano, 2007)

1. Tissue was fixed with 10% neutral buffered formaldehyde.
2. Part of the tissue was trimmed in such a way that the lesion we required to be included or not missed and to fit standard histological processing tissue cassettes (5 mm thickness).
3. Tissue Specimen Processing:

This process consists of fixation of tissue by formalin, dehydrating tissue by increasing alcohol concentrations, clearing of tissue by xylene and impregnation of tissue by paraffin wax.

Formalin-I for 2 hours, Formalin-II for 2 hours, 70% Alcohol for 1 hour, 95% Alcohol for 1 hour, 100% Alcohol-I for 1 hour, 100% Alcohol-II for 2 hours, 100% Alcohol-III for 2 hours Xylene I for 1:30 hours Xylene-II for 1:30 hours, Xylene-III for 1:30 hours, Paraffin-I for 2 hours and Paraffin-II for 3hours.

4. Embedding of processed tissue

Impregnated tissue was placed in a mould with labels and then fresh melted wax (54-60$^0$C) was poured and allowed to settle and solidify.

5. Sectioning

Tissue sections with a thickness of 4-5 microns were placed in a water bath to straighten the ribbon and then adhered to the surface of frost end and clear slide.

### Annex 2: Tissue staining procedures (Votano, 2007)

1. The sections were fixed on slides in xylene for 3 minutes.
2. Then the samples were transferred to absolute alcohol for 3 minutes.
3. Transferred to 80% alcohol for 2 minutes.
4. The samples were placed in 50% alcohol for 2 minutes.
5. The slide was washed in running tap water for 1 minute and placed in Harris's haematoxylin for 5-7 minutes.
6. Washed in running tap water for 30 seconds.
7. The excess dye was washed in 1% acid alcohol by continuous agitation for 15 seconds.
8. Washed in running tap water for 30 seconds.
9. Given 2-3 dips in ammonia water solution until tissues attained a blue color.

10. Washed in running tap water for 30 seconds.
11. Counter stained with eosin for 3-5 minutes.
12. Washed in running tap water for 30 seconds.
13. Dehydrated by keeping in increasing concentration of alcohol (2-3 minutes in 50%, 70%, 95% and absolute alcohol).
14. Cleared in xylene and mount with DPX.

**Annex 3: SPGA/EDTA (sucrose, phosphate, glutamate and albumin/ethylenediamine tetra-acetic acid) buffer preparation**

| | Concentration (M) | Weight |
|---|---|---|
| Sucrose | 0.2180 M | 7.462 g |
| Mono-potassium phosphate | 0.0038 M | 0.052 g |
| Di-potassium phosphate | 0.0072 M | 0.125 g |
| L-monosodium glutamate | 0.0049 M | 0.083 g |
| Bovine albumin powder | 1.0% | 1.000 g |
| EDTA | 0.2% | 0.200 g |
| Distilled water pH 6.5 | | 100 ml |

**Annex 4: Chicken embryo fibroblast cell preparation protocol**

1. Remove the embryos from 10-11 day-old eggs after having swabbed the tops of the shells with 70% alcohol.
2. Eviscerate the embryos, first cut off the head and limbs and place in PBS at $37^0$C.
3. Cut the remains of the embryos into small pieces in a Petri dish with scissors.
4. Transfer the tissue to a 150 ml flask and add 20 ml of PBS at $37^0$C.
5. Wash the tissue until the PBS becomes clear. Pour off the PBS and discard.
6. Add 20 ml of Trypsin solution at $37^0$ c swirl and allow the tissue to settle. Pour off the supernatant and discard.
7. Add 20 ml of Trypsin solution and place the flask on a magnetic stirrer in a bench incubator at $37^0$C. Stir for 15 minutes at a rate sufficient to give through mixing.
8. Decant the supernatant cell suspension into a universal bottle containing needed amount foetal calf serum and mix.

9. Repeat the procedures in steps 7 and 8.
10. Filter the cell suspension through double layer of sterile muslin.
11. Centrifuge the cell suspension at 600 rpm for 5 minutes.
12. Using a 10 ml pipette, re-suspend the cell pellets in 8 ml complete GMEM and pour into a 10 ml graduated centrifuge tube.
13. Centrifuge the cell suspension at 900 rpm for 5 minutes.
14. Using a 10 ml pipette, dilute the cells 1: 300 in complete GMEM (8 eggs should give approx. 1 ml of packed cells).
15. Dispense the cell suspension into the required vessels and incubate at $37^0$C (7.5 ml for 25 $cm^2$, 25 ml for 75 $cm^2$ and 50 ml for 162 $cm^2$ flasks.
16. Confluence should be obtained within 24 hours.

**Annex 5: Growth and maintenance media**

Growth medium consisted of GMEM supplemented with 10% tryptose phosphate broth (TPB), 0.63% of a 10% NaHCO3 solution, 1% of Antibiotic-Antimycotic Mixture 100X (Gibco, Grand Island, New York, USA) and 10 % foetal bovine serum (FBS). For CEF this medium will be replaced after 24 hours by the same medium but supplemented with only 2-5% FBS.

**Annex 6: Phosphate buffered saline (PBS) without calcium or magnesium**

| | |
|---|---|
| Sodium Phosphate Dibasic ($Na_2HPO_4$) | 1.60 gms |
| Potassium phosphate ($KH_2PO_4$) | 0.51 gms |
| Sodium Chloride (NaCl) | 7.30 gms |
| Double distilled water Sterilized by Autoclave | 1 liter |

## Annex 7: DNA extraction

### DNA extraction from feather follicles (Qiagen, Germany)

1. Hold a small spring with sterile forceps, and then cut with a sterile scissors 1 cm (4 pieces) from the tip of spring off on.
2. Pipette 180 µl in this buffer ATL and 20 µl proteinase K and incubate at $56^0$ c for 20 hours.
3. Vortex repeatedly and centrifuge briefly.
4. Add 200 µl buffer Al mix for 15 seconds and incubate the mixture for 10 minutes at $70^0$ c.
5. Centrifuge briefly.
6. Centrifuge the suspension briefly with centrifuge tubes and add 200 µl of molecular grade
Ethanol (96-100%) and mix for 15 seconds.
7. Apply this mixture to precipitate in to a QIAamp spin column. The column is then centrifuge for 1 minute at 8000 rpm.
8. Place the QIAamp spin column in a clean collection tube. Discard the collection tube with filtrate.
9. Carefully open to the QIAamp spin column and add 500 µl buffer AW1. The closed column is then centrifuge for 1 minute at 8000 rpm.
10. Place QIAamp spin column in a new collection tube and discard the filtrate.
11. Carefully open to the QIAamp spin column and add 500 µl buffer AW2.close the tube and centrifuge for 3 minutes at 14,000 rpm.
12. Place QIAamp spin column in a sterile reaction vessel and discard the collection tube with filtrate.
13. Pipette 200 µl of buffer AE in the QIAamp spin column. Perform 1 minute incubation at room temperature.
14. Centrifuge for 1 minute at 8000 rpm and retain the filtrate at $4^o$ c for 48 hours at the latest. Prolong storage at $-20^o$ c

**DNA extraction from tissue samples (Qiagen, Germany)**

1. Cut tissue (≤ 10mg spleen or ≤ 25mg other tissue) into small pieces and place in a 1.5 ml micro-centrifuge tube. Add 180 µl Buffer ATL. Add 20 µl proteinase K, mix by vortexing and incubate at 56° C until completely lysed. Fifteen seconds prior to proceeding to step 2, and also occasionally during incubation, vortex directly.
2. Add 200 µl Buffer AL. Mix thoroughly by vortexing. Incubate samples at $56^0$C for 10 minutes.
3. Add 200 µl ethanol (96-100%). Mix thoroughly by vortexing.
4. Pipet the mixture into a DNeasy Mini spin column placed in a 2 ml collection tube. Centrifuge at ≥ 6000 x g (8000 rpm) for 1 minute. Discard the flow-through and collection tube.
5. Place the spin column in a new 2 ml collection tube. Add 500 µl Buffer AW1. Centrifuge for 1 minute at ≥6000 x g. Discard the flow-through and collection tube.
6. Place the spin column in a new 2 ml collection tube. Add 500 µl Buffer AW2 and centrifuge for 3 minutes at 20,000 x g (14,000 rpm). Discard the flow-through and collection tube.
7. Transfer the spin column to a new 1.5 ml or 2 ml micro-centrifuge tube.
8. Elute the DNA by adding 50 µl Buffer AE to the center of the spin column membrane. Incubate for 1 minute at room temperature (15-25oC). Centrifuge for 1 minute at ≥ 6000 x.

**Annex 8: PCR Mix (Master Mix.) for MDV-PCR**

| Reagents | Volume of (µl) final concentration |
|---|---|
| Platinum PCR mix | 10 |
| Primer forward MDV-1F | 0.6 |
| Primer reverse MDV-1R | 0.6 |
| Probe MDV-1 | 0.8 |
| BSA 5 mg.ml- 1 | 1 |
| H2O | 2 |
| Sample DNA | 5 |
| Total volume (µl) | 20 |

**Annex 9: Real-time PCR thermal cycle program**

| Target ($^0$ c) | Hold (hh:mm:ss) |
|---|---|
| Activation/denaturation | |
| 1 cycle $95^0$ c | 00:15:00 |
| 45 amplification cycle | |
| $95^0$ c | 00:00:15 |
| $60^0$ c | 00:00:15 |
| $72^0$ c | 00:00:10 |
| Cool | |
| 1 cycle $40^0$ c | 00:05:00 |

**Annex 10: Inoculation of a suitable monolayer cell on CEF with the collected supernatants**

1. Select a monolayer cell culture with a confluence of >70% and remove the growth medium.
2. Wash the culture twice with PBS.
3. Inoculate the specimen suspension on the test flask and PBS with antibiotics and Antimycotic (VTM) on the negative control flask and incubate at 37o c for 60 minutes to allow the virus to absorb on to the cell culture (1ml for 25cm2 and 3ml for75cm2tissue culture flask).
4. Add maintenance medium (MEM with 2% FCS) and incubate the flask at 37o for appropriate time, 3-7 days. Keep also control flasks without any specimen inoculums.
5. Observe for the effect of virus action.

It is essential that each material be passed in cell culture at least three times before declaring any specimen negative. The presence of viruses can be detected by observing cytopathic effects.

**Annex 11: Microscopic evaluation**

Once the smear has been prepared, stained and dried, it was scanned at low magnification **(4-10X objective)** to determine if all areas of the smear were stained properly and if there was adequate cellularity for evaluation. When proper staining was assured and all areas of increased and/or unique cellularity are recognized, magnification is increased to the 10X or 40X objectives. An impression of the cellularity and cellular composition of the smear and of cell size were ascertained. Using the **40X** objective, nucleoli and chromatin patterns were discerned. Cell morphology was evaluated in detail with a **100X (oil-immersion)** objective.