

**Molecular Epidemiology of Marek's Disease Outbreak in Chickens in
Selected Areas of Mid Rift Valley of Ethiopia**



TEMAM GELETU NAGESO

A Thesis Submitted to the Department of Applied Biology in Partial
Fulfillment of the Requirement of the Award of Degree of Master of
Science in Applied Biology (Biotechnology)

School of Applied Natural Sciences

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Adama Science and Technology University

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Declaration

I hereby declare that this MSc thesis entitled “Molecular Epidemiology of Marek’s Disease Outbreak in Chickens in Selected Areas of Mid Rift Valley of Ethiopia” is my original work. That is, it has not been submitted for the award of any academic degree, diploma or certificate in any other university. All sources of materials that are used for this thesis have been duly acknowledged through citation.

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List of Acronyms and Abbreviation

AGIDT	Ager Gel Immune Diffusion Test
AGPT	Agar Gel Precipitation Test
CAM	Chorio Allantoic Membranes
CAV	Chickens Avian Virus
CD4	Cluster Differentiation
CNS	Central Nerve System
CPE	Cytopathic Effect
CSA	Central Statistics Agency
DPI	Day Post Infection
ELISA	Enzyme Linked Immune Sorbent Assay
FAT	Fluorescent Antibody Test
FFE	Feather Follicle Epithelium
GaHV	Gallid Herpes Virus
Ha	Alternative Hypothesis
HHV	Human Herpes Virus
Ho	Null Hypothesis
HSV	Herpes Simplex Virus
HVT	Herpes Virus of Turkey
ICTV	International Committee on Taxonomy of Virus
IPT	Immuno Peroxide Test
IR	Internal Repeat
LAT	Latency Associated Transcripts
LMF	Latency Maintaining Factor
MATSA_s	Marek's Associated Tumor Surface Antigens

MD	Marek's Disease
MDV	Marek's Disease Virus
mMDV	Mild Marek's Disease Virus
NAHDIC	National Animal Health Diagnosis and Investigation Center
OIE	Organization for Animal Health
P	Probability Value
PCR	Polymerase Chain Reaction
RT-qPCR	Quantitative Real Time Polymerase Chain Reaction
T	Tegument
TH	Thymus Helper
TR	Terminal Repeat
UL	Unique Long
US	Unique Short
USD	United States Dollar
vMDV	Virulent Marek's Disease Virus
vvMDV	Very Virulent Marek's Disease Virus
vv+ MDV	Very Virulent Plus Marek's Disease Virus
WAHID	World Health Information Database

Abstract

Marek's disease (MD), caused by Marek's Disease virus (MDV) is a highly contagious oncogenic and neuropathic disease of chickens characterized by development of CD4⁺T cell lymphomas as well as infiltration of nerves and visceral organ by lymphocytes. It is a major threat to the poultry industry in many countries of the world including Ethiopia. The aim of this study was to conduct an outbreak based cross-sectional investigation of MD caused by *Gallid Alphaherpes virus type 2* (GaHV-2) by molecular tool February 2020 to November 2020 in chickens from selected areas in the Mid Rift Valley of Ethiopia. For this purpose a total of 82 samples pooled of five feather follicle from chickens were collected and DNA was extracted at National Animal Health Diagnosis and Investigation Center (NAHDIC), Sabata. Of the total samples tested for Meq genes using RT-qPCR, 8 (9.76%) samples were found positive for MDV. The prevalence of MD in male was found to be 7.84% and that in female was 10.62%. On the other hand, the prevalence of MD was higher in Adult (12%), compared to old (10.53%) and young chickens (4.44%). The prevalence of MD in backyard 16.23% and in poultry farm 14.13%. From the overall prevalence of 9.76%, the highest score was recorded in Bishoftu (12%) and no positive case was detected in Batu while intermediate (9.52%) score were obtained from Hawasa. In general, the present study provide important information on epidemiology of MDV in most poultry farm and backyard in the middle Rift Valley of Ethiopia, and highlight the importance of implementing surveillances and biosecurity in backyard and poultry farm.

Key words: *Marek's Disease Virus, Meq gene, Mild Rift Valley, RT-PCR, Outbreak.*

1. INTRODUCTION

1.1. BACKGROUND OF THE STUDY

In Ethiopia chickens breeding are the most widespread and almost every rural family owns chickens, which provide a valuable source of family protein and income. The total chickens population in the country is estimated to be 60 million with native chickens representing 90%, hybrid chickens 6.75% and exotic breeds 2.25% (Dana, 2019). In rural parts of Ethiopia farmers keep poultry for various purpose which include hatching, sale, home consumption, sacrifices (healing ceremonies) and gifts (Teklewold *et al.*, 2006). The most dominant chickens types reared in Ethiopia are local ecotypes, which show a large variation in body position, plumage color, comb type and productivity (Halima *et al.*, 2007). Though rural poultry constitutes about 99% of the national poultry production in the country, the system has constraints which restrict its potential. These constraints are low inputs of feeding, poor management, the presence of diseases of various natures and lack of appropriate selection and breeding program (Yami, 1995; Ashenafi, 2000; Dessie and Ogle, 2001).

Among diseases that affect chickens production in Ethiopia, Marek's disease (MD) is one that is widely distributed. MD is a highly contagious and economically important oncogenic or paralytic viral disease of poultry. The disease is becoming a serious problem of the poultry industry of Ethiopia. MD is caused by Marek's disease virus (MDV), an alpha-herpesvirus that is ubiquitous to poultry, spreads horizontally that means an infection agents spread from one individual to other, and can cause tumor formation in visceral tissues (Bavananthasivam *et al.*, 2018). MD was first reported in 1907 by a veterinarian named József Marek, after whom the disease was named (Marek, 1907). The first cases of MD were reported in the US in 1914 (Witter and Shat, 2003). During the late twentieth decade, there were increases in the severity of MD, but in general the disease has been controlled successfully by the use of vaccines.

The genomic structure of Marek's disease virus DNA was found to be similar to that of herpes simplex virus types 1 and 2. A long unique region (75 x 10⁶ MW, located at 10 x 10⁶ to 85 x 10⁶ MW [10-85] from the left end of the genome), which was subdivided into segment 1 (22 x 10⁶ MW, located at 10-32) and segment 2 (51 x 10⁶ MW, located at 34-85) by direct repeats (32-34), was flanked by a long terminal region (10 x 10⁶ MW, located at 0-10) and a long

inverted region (10 x 106 MW, located at 85-95). A short unique region (8 x 106 MW, located at 103-111) was flanked by a short terminal region (8 x 106 MW, located at 111-119) and a short inverted region (8 x 106 MW, located at 95-103). The direct repeat fragments (0.9 x 106) could be isolated by cleavage with SmaI. The right terminal end was found to be heterogenous (Kunihiko *et al.*, 1984).

Lobago and Woldemeskel (2003) conducted a study on an outbreak of Marek's disease in a commercial poultry farm in central Ethiopia reporting a mortality rate of 46% for the first 14 weeks of the outbreak. According to Duguma *et al.* (2005) the magnitudes of morbidity and mortality, was reported to be 67.9% (340/503) and 66.2% (333/503), respectively, on indigenous chickens in Ethiopia which were nearly equal, indicating that MD is highly fatal (97.9%) to the local breeds. On the other hand MD vaccination significantly increased the survival rate of the local breeds (Duguma *et al.*, 2006).

This indicates the dramatic effect of MD vaccination on the survival of the indigenous chickens and vaccination could be a vital instrument to combat the threat by MD. However, despite the growing importance of MD vaccine in Ethiopia, it is being availed by importation from India. Starting from 2013 G.C, Ethiopia imported 1.8 million doses of MD vaccine (HVT FC-126 strain) and distributed for customers. Nevertheless, still there are a lot of MD outbreak reports in different regions of the country where imported MD vaccines were still in use. These vaccines are produced from MDV strains isolated from different production systems and because of this they may not be as effective as they are supposed to be (Witter, 1992; Gimeno, 2008). Duguma *et al.* (2006) also underlined the need for development of MD vaccines from local MDV strains for securing the efficacy of the vaccines being used.

Imported vaccines are acquired from elsewhere via foreign currency and with higher transportation cost, and this makes them to be more costly. Development and production of effective MD vaccine locally via isolation of MDV virus strains from the local production systems is mandatory. However, previously no study has been conducted in Ethiopia by Real Time quantitative polymerase chain reaction to separate field strain from vaccinated strain and to determine the prevalence of MD at molecular level.

1.2. STATEMENT OF THE PROBLEM

Different study done on chickens in Ethiopia found out many diseases in the chickens; from that disease Marek's disease is one of disease found in different parts of country. There are different factors that play a role in the prevalence of Marek's disease such as lack of routine sanitary and hygienic practices, backward waste disposal, weak housing management, lack of appropriate transportation for poultry and poultry related products, inadequate feed and water in terms of quality and quantity, lack of insurance and finance support, inadequate veterinary service, and inadequate vaccination practices of chickens. Despite live and inactive lentogenic vaccines are used in Ethiopia; however, there are complaints with these vaccines as not producing active immunity to block MDV, the method of diagnosis, and lack of sufficient and organized data about the disease. Because of those aforementioned reasons, Marek's disease represents the most severe poultry disease responsible for the most economic losses across the country (Tadelle *et al.*, 2003).

There is a scarcity of information on the molecular status of the virus in the village and commercial chickens in the study area. Thus, there is a need for sufficient and accurate data that are produced by molecular diagnosis method to design effective controlling and eradication strategies for MD.

1.3. OBJECTIVE OF THE STUDY

1.3.1. GENERAL OBJECTIVE

- To screen the outbreak of Marek's disease virus by molecular techniques in the chicken's population found in poultry farm and backyard in Mid Rift Valley of Ethiopia.

1.3.2. SPECIFIC OBJECTIVES

The specific objective of this study

- To determine the prevalence of Marek's disease in different age, sex, in back yard chickens and commercial poultry farm found in Mid Rift Valley of Ethiopia
- To quantify outbreak of Marek's disease virus by using RT-qPCR in selected geographical areas in Mid Rift Valley of Ethiopia.

1.4. SIGNIFICANCE OF THE STUDY

Marek's disease is economically important, due to the fact that it causes high morbidity and mortality rates, reduces egg production, deteriorates egg quality, and impairs life performance. The importance and impact of a Marek's disease virus are directly related to its virulence. Major efforts to reduce the outbreak and prevalence of Marek's disease have been undertaken in the last decades (Berhan, 2014). The main challenge in Marek's disease control is the lack of updated and accurate data that are important for diagnosis, vaccine production, and developing a control strategy. This study addresses the more reliable and updated information about the current status of MD and fills the gap of information. Enhancing the knowledge of Marek's disease causing strain of virus help to control and reduce the expansion of the disease and increase the production of the chickens in the country. It is also used to develop native vaccine to the chickens for the possible solution of the Marek's disease virus that make economic crisis in our country and to explain the current status for the perspective body about the disease.

1.5. SCOPE OF STUDY

The principal concern of this study was to investigate the outbreak of Marek's disease in chickens in selected areas in Mild Rift Valley of Ethiopia. Due to time and financial constraints, the current study could not cover the country wide samples to know the whole picture of the situation in Ethiopia.

1.6. HYPOTHESES

- H_0 = There is similarity in distribution of Marek's disease in different geographic area of Ethiopia.
- H_a = There is difference in distribution of Marek's disease in different geographic area of Ethiopia.

2. LITERATURE REVIEW

2.1. DESCRIPTION OF THE DISEASE

Marek's disease (MD), a lymphoproliferative disease of domestic chickens less commonly, turkeys and quails, is caused by an "alphaherpesvirus". MD viruses cause clinical manifestations can be numerous and varied, including paralysis, skin lesions, atrophy of the thymus and bursa of Fabricius immunosuppression and high mortality (Calnek, 1985; Hennig *et al.*, 2003; Schat, 2008b). It is caused by Marek's disease virus (MDV), an *Alphaherpesvirus*, and is characterized by T cell lymphomas, polyneuritis, immunosuppression and, rarely, atherosclerosis (Calnek, 2001). Three MDV serotypes have been identified, out of which only serotype 1 (MDV-1) is pathogenic, whereas MDV serotype 2 (MDV-2) and serotype 3 (herpesvirus of turkeys, HVT) do not cause MD. MDV infection results in the establishment of a latent infection, which is common in *Alphaherpesvirinae*; however, MDV has unique properties when compared with other members of this virus subfamily because it can cause tumours and integrate its DNA into the host cell genome (Baigent and Davison, 2004).

Birds get infected by inhalation of infected dust from the poultry houses, and following a complex life cycle, the virus is shed from the feather follicle of infected birds. Mortality rates can be very high in susceptible birds. MD commonly appears in 3 to 4 weeks old chickens and gradually builds to a peak between 12 and 30 weeks of age. Poultry may be unable to lay eggs or may even die. Affected birds are more susceptible to other infectious diseases (Calnek, 2001).

2.2. HISTORY OF MAREK'S DISEASE

The story starts with a publication in 1907 by Dr. Joseph Marek, an eminent veterinary clinician, pathologist, Professor and Head of the Department of Veterinary Medicine at the Royal Hungarian Veterinary School in Budapest. He described the disease as a "neuritis interstitialis" or a "polyneuritis" (Biggs, 1967). Pappenheimer *et al.* (1926) reviewed the literature of the period prior to their studies and considered the 1907 report by Marek from Hungary to be the first account of the disease. According to Bigg (1967), Marek's disease was first recorded in Great Britain by Galloway in 1929. Several outbreaks of the disease were reported by Kaupp (1921) in the United States as MD was discovered. It was also subsequently noted in many other countries. Initially the disease was described as 'polyneuritis' while studying four adult cockerels, which were affected by paralysis of wings and legs. The first report of Marek's disease in United Kingdom was made

in a meeting at Royal society of medicine by Galloway in 1929. For another 40 years the etiology, nature of lesions and transmission was argued until period 1962-1970 when herpesvirus was isolated from turkeys and vaccine being produced. Before introduction of vaccination in 1970, Marek's disease caused annual losses of 150 million USD in United States and 40 million USD in United Kingdom (Fehler, 2001).

2.3. SEROTYPES AND PATHOTYPES OF MDV

Three serotypes of MDV and related Herpes viruses have been defined. Serotype 1 includes all the pathogenic or oncogenic strains of these viruses. Serotype 2 includes naturally non-attenuated strains of MDV. Serotype 3 includes turkey herpesvirus (HVT), the non-oncogenic MDV related virus isolated from turkey (table 1). New pathotypes have been emerging indicating continuous evolution of MDV towards greater virulence. Serotype 1 MDVs could be further classified into four pathotypes, including mild (m), virulent (v), very virulent (vv) and very virulent plus (vv+) strains (Sharma and Witter, 2003). The main basis for the pathotypes classification is the ability to cause disease in chickens immunized with increasingly effective vaccines (Shamblin *et al.*, 2004).

Table 1: Classification of MDV serotypes and their representative strains

MDV serotype	Characters	Pathotypes or strains	Reference
Serotype 1	Pathogenic or oncogenic strains as well as attenuated strain of these virus	Very virulent plus (VV+):648A Very virulent (VV):Md/5, Md/11, Ala-8,RB-1B Virulent (V): HPRS-16, JM. GA Mild (M) virulent: HPRS-B14, Conn Weakly virulent: CU-2, CVI-988	(Kenenet, 2013)
Serotype 2	Naturally non-pathogenic, non-oncogenic or avirulent strain	SB-1, HPRS-24, 301B/1, HN-1	
Serotype 3	Naturally avirulent strain, non-oncogenic	HVT(FC126, PB1) Herpes virus of Turkey	

MDV infection results in the establishment of a latent infection, which is common in *Alphaherpesvirinae*; however, MDV has unique properties when compared with other members of this virus subfamily because it can cause tumours and integrate its DNA into the host cell genome (Baigent and Davison, 2004).

2.3.1. PATHOTYPES AND EVOLUTION OF VIRULENCE

The increase in virulence of MDV over the past 50 years is of major significance. The shift from mMDV to vMDV strains in the late 1950s, the shift from vMDV to vvMDV in the late 1970s and, more recently, the appearance of the putative vv+ pathotype in the early 1990s have each resulted in the potential for greater disease losses that have persisted until introduction of a more effective vaccine (Witter, 1998b).

Rosenberger and Coworkers (1997) have noted instances in which the isolated MDV strains of high virulence appeared to have increased pathogenicity for adult chickens, a finding consistent with that reported by Witter (1996). The conditions that favor evolution of MDV towards greater virulence are not known but are probably created in part by vaccination with increasingly effective products. As vaccine immunity is compromised by factors such as early exposure or immunosuppressive stress, mutant clones have an increased opportunity to selectively multiply and to be seeded in the environment. Because vaccines are the major barrier to the disease, single point mutations may be sufficient to result in the emergence of a mutant strain (Renz *et al.*, 2012).

2.4. AETIOLOGY

The causative agent of the Marek's diseases is Marek's Disease Virus (MDV) and as per the recent classification by the International Committee on Taxonomy of Viruses (ICTV, 2011). It is placed in Order *Herpesvirales*, family *Herpesviridae*, subfamily *Alphaherpesvirinae*, genus *Mardivirus* and species *Gallid Alpha Herpesvirus 2 (GaHV-2)*. Due to its lymphotropic nature, MDV was originally classified in the family *Herpesviridae* as a member of subfamily *Gammapherpesvirinae*. However, on the basis of genomic organization, MDV is currently classified with the viruses of subfamily *Alphaherpesvirinae*, genus *Mardivirus*, and species *Gallid Herpesvirus 2 (GaHV-2)*. MDV strains (MDVs) had become increasingly virulent since the 1960s (Schat and Nair, 2008; ICTV, 2011).

2.5. **HERPESVIRIDAE FAMILY**

Herpesviridae is a large family of DNA viruses that cause diseases in animals, including humans (Baigent and Davison, 2004). The members of this family are also known as herpes viruses (Fenner *et al.*, 2011). The family name is derived from the Greek word *herpein* ("to creep"), referring to the latent, recurring infections typical of this group of viruses. *Herpesviridae* can cause latent or lytic infections. The family consists of 21 viruses species divided into three subfamily and six genera (Lee *et al.*, 2008).

2.6. **MAREK'S DISEASE VIRUS**

2.6.1. **VIRUS MORPHOLOGY**

All *Herpesviruses* share a common structure and all herpesviruses are composed of relatively large double-stranded, linear DNA genomes encoding 100-200 genes enclosed within an icosahedral protein cage called the capsid which is itself wrapped in a protein layer called the Tegument containing both viral proteins and viral mRNAs as well as lipid bilayer membrane called the envelope (Stamilla *et al.*, 2020). This whole particle is known as a virion. Enveloped, spherical to pleomorphic, 120- 200 nm in diameter T=16 icosahedral symmetry. The capsid consists of 162 capsomers and is surrounded by an amorphous tegument. Glycoproteins complexes are embedded in the lipid envelope (Lee *et al.*, 2000).

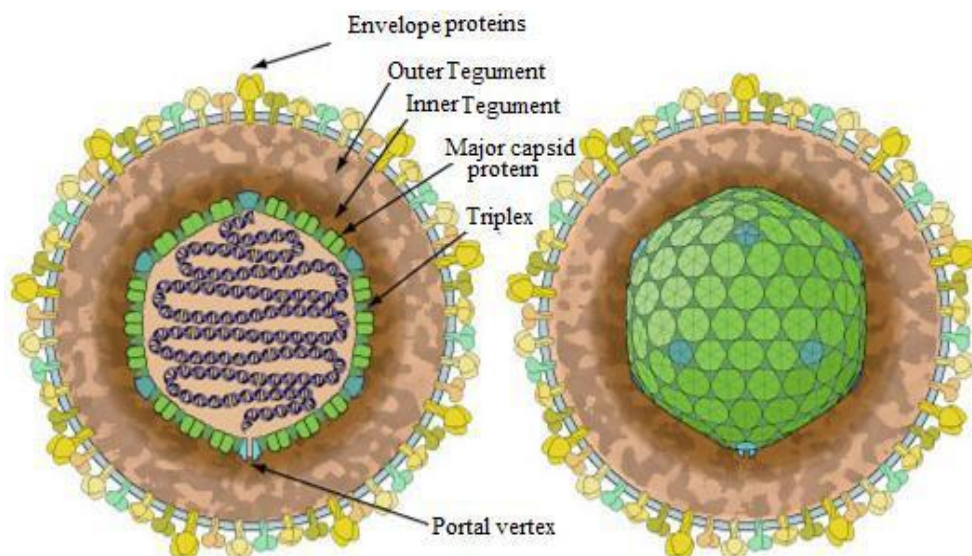


Figure 1: Virion structure of herpesvirus (Source: Viral zone, 2012).

2.6.2. GENOME ORGANIZATION

The MDV genome is a linear double-stranded DNA of approximately 160-180 kbp in size, which contains a unique long (UL) sequence and a unique short (US) sequence, both flanked with terminal repeat (TR) and internal repeat (IR) sequences own its structure. This genome belongs to group E, like the human herpesvirus 1 (HHV-1). The MDV genome contains about 100 open reading frames and encodes more than 70 genes, most of which have orthologous equivalents in other *alpha herpes viruses* (e.g., tegument genes like UL36 [VP1/2], the largest ORF in the genome, UL47 [VP13/14] and UL49 [VP22] or capsid genes like UL19 [VP5]). However, some genes are specific to MDV, such as the gene encoding Meq oncoprotein or pp38 phosphoprotein (Tulman *et al.*, 2000).

The nucleotide sequences of serotype 1 (oncogenic) strains of MDV, serotype 2 (non-oncogenic) strains, and serotype 3 (herpesvirus of turkeys) strain have been published. The ICP4 gene is encoded in the inverted repeat flanking the unique short region. The coding sequence, which is 4,245 nucleotides, is highly conserved among various strains. There are many transcriptional regulatory sites upstream and downstream of the ICP4 translational start site (Lee *et al.*, 2000). A long open reading frame is positioned from 5' to frame with the conventional ICP4 coding sequence. Also, the ICP4 promoter region in MDV1 CVI988 strain has insertions and duplications compared to ICP4 promoters of other MDV1 strains. The function of ICP4 in MDV is still not fully understood.

Transfection of the ICP4 gene into lymphoblastoid cells increased the expression of pp38 and pp24 genes, suggesting that the protein is a transactivator presented the first complete genomic sequence, with analysis of a very virulent strain of MDV serotype-1 Md5 (Izumiya *et al.*, 2001). The genome ICP4 is 177, 874 bp and was predicted to encode 103 proteins. MDV1 is a coline with the prototypic herpes simplex virus type 1 (HSV- 1) within the unique long (UL) region, and it is most similar at the amino acid level to MDV2, herpesvirus in turkeys (HVT), and non-avian herpesviruses, equine herpesviruses 1 and 4. MDV1 encodes 55 HSY-1 UL regions homologous together with six additional UL proteins that are absent in non-avian herpesviruses. The unique short (US) region is collinear with and has greater than 99% nucleotide identity to that of the MDV strain GA. However, an extra nucleotide sequence at the Md5 US/short terminal repeat boundary results in a shorter US region and the presence of a second gene (encoding MDV 097) similar to

SORF2 gene Md5, like HVT, encodes an ICP4 homologous that contains a 900-amino acid-amino-terminal extension not found in other herpesviruses. Md5 contains only two copies of the 132 bp repeat, which has previously been associated with viral attenuation and loss of oncogenicity (Tulman *et al.*, 2000).



Figure 2: Genome organization of Marek's disease herpes virus. Unique long (UL) and Short (US) regions are flanked by inverted and terminal repeat long and short regions (IRL, IRS, IRL, and TRS) (Source: Tulman *et al.*, 2000).

2.7. PATHOGENESIS

The Cornell Model describes the pathogenesis of MD with four phases: early cytolytic, latency, secondary cytolytic, and transformation (Calnek, 2001).

2.7.1. EARLY CYTOLYTIC PHASE

MDV is taken into chickens through inhalation of infectious dander, and enters the circulation via phagocytosis by the lung epithelium, which transfer the virus to recruited macrophages and B-cells (Cui *et al.*, 2004). The virus genome is detectable in primary and secondary lymphoid tissues, bursa of Fabricius, thymus, spleen and cloaca tonsils, as early as 2-7 dpi, accompanied by expression of MDV early protein pp38 and lymphocytolysis with necrosis and infiltration of inflammatory cells (Calnek *et al.*, 1984). Macrophages have been shown to support some level of lytic infection and increased infection of macrophages has been seen with higher virulent MDVs (e.g. C12/130). The main target cells of this early lytic phase are B cells, which are substantially eliminated in bursa of Fabrics and circulation. However, B cells in the spleen are not essential for MDV replication, as surgical removal of spleens either had no effect on the appearance of latently-infected blood lymphocytes, nor did it abolish lymphoma development (Schat, 1987).

During this lytic phase of infection, the activation of T cells, particularly CD4⁺ cells, is observed. During this phase, no infectious cell-free virus is produced, and it is still unknown how the virus spreads between cells. However, Jacob, (2015) shows that gH/gL complex is essential for spreading between uninfected cells, and others have demonstrated that gB, gE/gI, gM/gN and VP22, are essential for MDV cell-to-cell spreading (Dong, 2011).

2.7.2. LATENCY

Following the early cytolytic phase of infection, MDV establishes latency in T-cells, primarily CD4⁺ T-cells. The definition of herpesvirus latency is the persistence of the viral genome in infected cells without the production of infectious virus. By 6-7 dpi, the MDV lytic antigen expression subsides in lymphoid organs and the switch from early lytic phase to latency phase starts (Baigent and Davison, 2004; Cui *et al.*, 2004). The host immune response to lytic infection has been shown to play an essential role in switching from cytolysis to latency. The role of host factors in latency is supported by the findings that immunosuppression prior to infection with MDV leads to prolonged early lytic infection, and chemically induced immunosuppression after latency leads to reactivation and cytolytic infection. Latency maintaining factor (LMF) and host cytokines, as well as soluble mediators (nitric oxide, NO), are involved in maintaining latency (Yang *et al.*, 2020).

In addition, unlike the early lytic phase, the predominant infected cells in latency are CD4⁺ TCR $\alpha\beta$ ⁺ T cells, which can be detected as early as 3 dpi. During latency, transcription of the viral genome is limited to latency-associated transcripts (LATs), a complex family of spliced RNAs localizing to nucleus that is abundant in MDV transformed cells, but reduced upon MDV reactivation. Meq also plays a role in maintaining latency by blocking apoptosis of CD4⁺ cells transactivating latent gene expression, and suppressing the promoters of MDV lytic genes ICP4 and pp38/pp14 (Nair, 2013).

2.7.3. SECONDARY CYTOLYTIC PHASE

The virus undergoes reactivation and another semi-productive infection and cytolysis cycle approximately 3 - 4 weeks post-infection, coincident with the immunosuppression associated with proliferation of latently-infected CD4⁺ T-cells. Although it is still unknown what causes the virus switch from latency to secondary cytolytic phase, it has been confirmed that once the virus is reactivated from latency, the virus replicates within the feather follicle epithelium (FEE) and will be shed to the environment in the dander, where it can remain infectious essentially indefinitely (Barrow *et al.*, 2003). Expression of glycoprotein D (gD) was believed to be associated with virus shedding, as it is only expressed in FFE in vivo. However interruption of the gD-encoding gene (US6) does not prevent horizontal transmission. More recently, glycoprotein C (gC) and the protein kinase encoded by the UL13 gene was found to be essential for horizontal transmission of MDV. However, the mechanisms regulating MDV productive infection at this site are currently unknown (Abdul-Careem *et al.*, 2008).

2.7.4. TRANSFORMATION

During the secondary cytolytic phase, the transformation phase of infection becomes apparent, in which latently-infected CD4⁺ T cells proliferate and give rise to lymphomas. The spleen is a primary site for lymphomas formation; however, it is not essential for this, because splenectomized birds still develop tumors (Lian *et al.*, 2012). Lymphomas have been observed in multiple organs, including the spleen, kidney, liver, intestine, and skin. MDV-transformed cells are a mixed population of T-lymphocytes expressing CD30, Marek's EcoRI-Q-encoded protein (Meq), MHC-II, and Marek's associated tumor surface antigens (MATSAs). CD30 is a host-encoded surface antigen, which is over expressed in EBV associated human lymphomas such as Hodgkin's lymphoma, in which it is called the Reed-Sternberg antigen (Ross, 1999).

The proposed function of CD30 in tumor formation is that; it contributes to polarization of the host immune response to a TH2 or humoral response, an ineffectual response in anti-tumor immunity. Thus, MDV-transformed T-cells appear to have a TH3 or T-regulatory (T-reg) immune-phenotype. The relationship between latently infected cells and transformed cells is unclear, but it is confirmed that the episomal and integrated copies of MDV genome co-exist with integrated forms being much more common (Katneni, 2015).

The site of integration is randomly distributed and transformation is associated with the integration sites near telomeres of the host's mini-chromosomes and larger chromosomes. Integration of the MDV genome may inhibit the expression of cytolytic gene expression and/or enhance the latency and transformation associated gene transcription. In a recently described model for MDV latency, the MDV genome was found to integrate in an REV transformed T-cell line and remain hypomethylated, indicating that DNA methylation is not directly involved in the silencing of lytic-phase genes during latency figure 3 (Katneni, 2015).

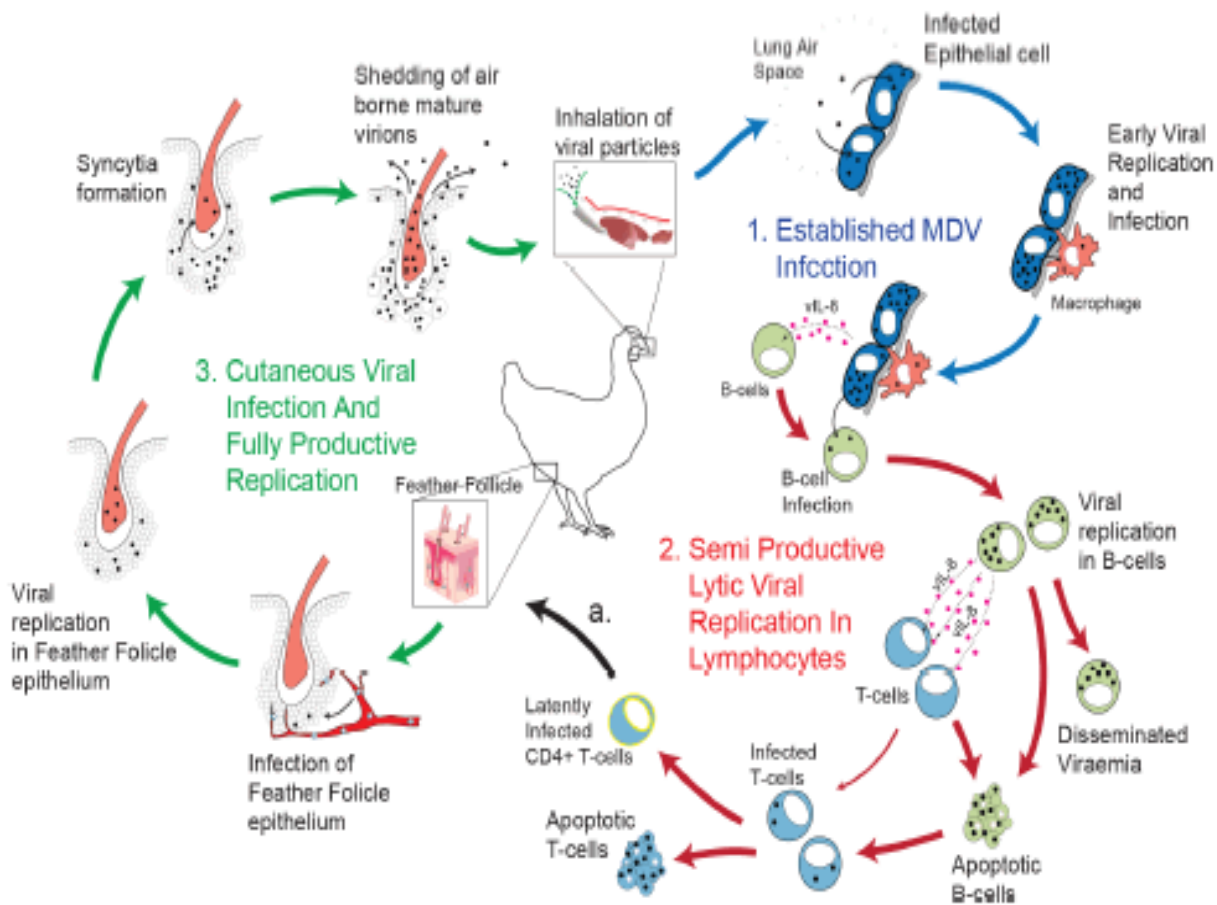


Figure 3: Pathogenesis of MD. Birds get infected by the inhalation of infectious virus shed from the feather follicle epithelium (FFE). After initial replication in the lungs, the virus replicates in the lymphoid organs. B-cells (B) and macrophages (M) undergo a lytic infection, resulting in the activation of T-cells, which are targeted by the virus. T-cells are transformed by the virus to produce tumors in different organs. Infected T-cells carry the infection to the feather follicle epithelium (Source: Boodhoo *et al.*, 2016).

2.7.5. MOLECULAR PATHOGENESIS OF MAREK'S DISEASE

The virus-cell relationship of MDV is complex, and cell-free virus is recovered only from the feather follicle epithelium, while in all other tissues, as well as in cell culture, the virus is mostly cell-associated. The mechanisms of entry of cell-free virus into cells have been little studied and the virus receptors have not been identified (Venugopal and Payne, 1995). Lack of knowledge of receptors for MDV entry will certainly limit the understanding of the cascade of events in the early pathogenic processes. After an early cytolytic infection, the virus induces lymphomas in T-cells. These cells are latently infected with the virus, but very few viral transcripts or proteins are detectable. Although there are indications that some of these virus-encoded transcripts may be involved in tumourigenesis, the exact nature of the virus cell interaction contributing towards the transformed phenotype is not completely understood (Woźniakowski and Samorek-Salamonowicz, 2014b) .

The MDV genome codes several unique proteins, some of which have been associated with the oncogenicity of the virus. Meq is the most extensively studied gene of MDV and it codes for a protein that shares significant homology to the family of transcriptional factors. Meq is consistently expressed in all MDV transformed cells, suggesting that it may play an important role in transformation. Pp38 is a phosphoprotein expressed in both lytically infected and tumor cells. The function of this protein is still not clear but has been suggested to be involved in the maintenance of transformation. Understanding the role of these proteins play in oncogenesis requires the introduction of mutations in the viral genome (Teng *et al.*, 2011).

2.8. TRANSMISSION

Marek's disease virus is shed in dead skin and feather follicle epithelial cells, where enveloped infectious virions exit from the body that added to the dust found in chickens houses, this disease is spread horizontally but, it is not spread vertically from chickens to eggs. MDV is spread easy by bird-to-bird contact, and contact with infected dust and dander and indirect contact with infected chickens, premises litter, and chopped feathers and airborne route to the environment and to other chickens as summarized on figure 4 (Jarosinski *et al.*, 2007). Very soon after infection of the respiratory tract, cell associated viremia can be detected in the blood, reaching a peak about eight days later (Carrozza *et al.*, 1973). Macrophages carry and distribute MDV all over the body

infecting sensitive cells and causing lymphocyte transformations. Once the virus is shed into the environment, it can remain infectious for many months (Nazerian *et al.*, 1968).

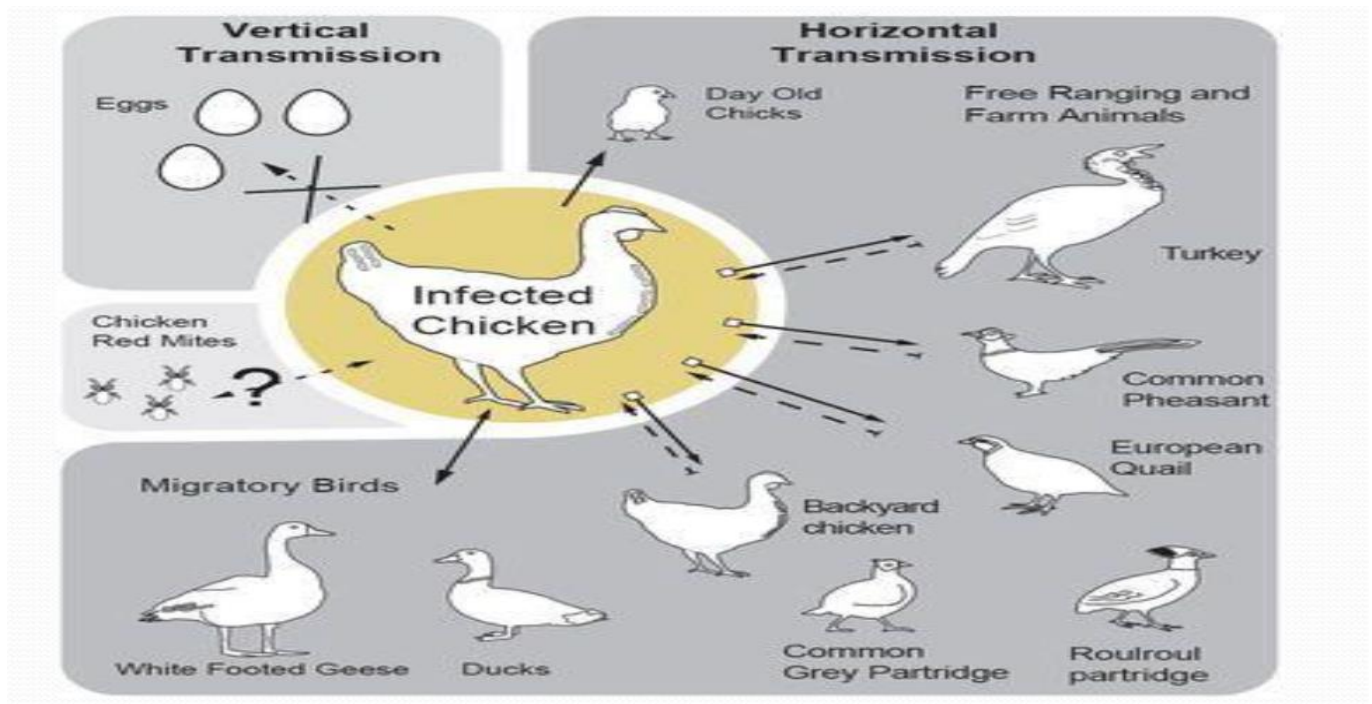


Figure 4: Model for horizontal and vertical transmission of MDV between avian species. It has been confirmed that MDV cannot be transmitted vertically from layer hens to eggs but day old chicks become infected in broiler production housing systems from shedders. Free ranging and farm animals consist of turkeys, common pheasant, common grey partridge and European quail. Migratory birds that have been confirmed positive are ducks and white footed geese. Although these birds become infected and are carriers, what has not been identified is their role in viral virulence factors as well as ability to infect broiler production system. Furthermore, the presence of chicken red mites has yet to be confirmed in broiler production system which could contribute to viral persistence (Source: Boodhoo *et al.*, 2016).

2.9. DIAGNOSIS OF MAREK'S DISEASE

Primary diagnosis is based on age, clinical signs, history and gross and microscopic lesions. Diagnosis of MD is easier in general in chickens younger than 14 weeks of age. Grossly, the disease is characterized by paralysis of legs, wings and neck, and tumour nodules in visceral organs depending upon the tissue or organs involved (Gimeno *et al.*, 2016). Other observations include grey eye (iris) or irregular pupil, vision impairment, blindness, skin lesions and immunosuppression (Kumar *et al.*, 2016).

2.9.1. CONVENTIONAL METHOD OF MAREK'S DISEASE DIAGNOSIS

Laboratory conformation is done by virus isolation in susceptible chicks, embryonated eggs and tissue cultures and subsequent identification (Othman and Aklilu, 2019). MDV can be isolated in chicken's embryos by yolk sac route (4-5 days embryo) and later examining their chorio-allantoic membranes (CAM) on 18th day of incubation for the pock lesions'. Infected embryos also show atrophy of muscles and curling. MD virus can also be isolated from chickens kidney, cells culture and chickens fibroblast cell culture systems. After 5-14 days, plaque formation or cytopathic effects (CPE) are observed in cell culture. Serotype can be confirmed by using specific monoclonal antibodies (Boodhoo *et al.*, 2016).

Diagnosis of lymphoid tumors in poultry is complicated due to multiple etiological agents capable of causing very similar tumors. It is not uncommon that more than one avian tumor virus can be present in a chickens, (Cortes *et al.*, 2011) thus one must consider both the diagnosis of the disease/tumors (pathological diagnosis) and of the virus (etiological diagnosis) (Zelník, 2004). A step-wise process has been proposed for diagnosis of Marek's disease which includes (1) history, epidemiology, clinical observations and gross necropsy, (2) characteristics of the tumor cell, and (3) virological characteristics (Gimeno *et al.*, 2005).

Key clinical signs as well as gross and microscopic features that are most useful for differentiating Marek's disease from Lymphoid Leukosis and Reticuloendotheliosis include (1) Age: MD can affect birds at any age, including <16 weeks of age; (2) Clinical signs: Frequent wing and leg paralysis; (3) Incidence: >5% in unvaccinated flocks; (4) Potential nerve enlargement; (5) Interfollicular tumors in the Bursa of Fabricius; (6) CNS involvement; (7) Lymphoid proliferation

in skin and feather follicles; (8) Pleomorphic lymphoid cells in nerves and tumors; (9) T-cell lymphomas (Siccardi and Burmester, 1970).

In addition to gross pathology and histology, other advanced procedures used for a definitive diagnosis of Marek's disease include immunohistochemistry to identify cell type and virus-specific antigens, standard and quantitative PCR for identification of the virus, virus isolation to confirm infections and serology to confirm/exclude infections (Cortes *et al.*, 2009).

2.9.2. RECENT ADVANCES IN MAREK'S DISEASE DIAGNOSIS

Viral antigen can be detected in feather tips, follicle epithelium and infected lymphoid tissue can be diagnosed by Agar Gel Precipitation Test (AGTP), Fluorescent Antibody Technique (FAT), Immunoperoxide Test (IPT) and Enzyme-linked Immuno sorbent Assay (ELISA) (Shat and Nair, 2008). Immunohistochemistry can be effectively used to diagnosis MDV proteins especially in all MD tumors (Kumar *et al.*, 2016).

2.9.2.1. POLYMERASE CHAIN REACTION

A PCR method was developed for the detection of Marek's disease (MD) and reticulo endotheliosis (RE) in formalin fixed paraffin embedded (FFPE) tissue, and for the detection of MD in tissues only preserve in 10% neutral buffered formalin (Upadhayay and Ewam, 2012). MD virus (MDV) and RE virus pro-viral DNA were detected in FFPE tissues stored for over 20 years (Fenner *et al.*, 2011). The PCR of formalin fixed and FFPE tissues is a simple and valuable tools that can be used to identify MD and RE infection (Davidson *et al.*, 1995). The full length genomic sequences of MDV 1 (Gen-Bank accession numbers: strain Md5; AF243438, GA; AF147806, Mdl 1; AY510475, CV1988; DQ530348), MDV2 (Gen-Bank accession number: strain SB-1; HQ840738, HPRS24; AB04935 And MDV 3 (Gen-Bank accession number: strain FC126; AF291866) are available now (Izumiya *et al.*, 2001).

This enables the PCR-based diagnostic methods for serotype specific detection of MDV (Islam *et al.*, 2006). PCR tests enabling differentiation of oncogenic and non-oncogenic strains of MDV serotype and MDV vaccine strains of serotype 2 and 3 (Becker *et al.*, 1992; Zhu *et al.*, 1992).

2.9.2.1.1. NESTED POLYMERASE CHAIN REACTION

Specific detection of Meq oncogene of MDV 1 in infected spleen cells, feather tips and peripheral blood mononuclear cells that can be tested by nested PCR have been developed (Murata *et al.*, 2007). To develop a diagnostic method to differentiate highly virulent strain of MDV from the attenuated MDV vaccine strain, CVI988 which is widely used, nested polymerase chain reaction (PCR) was performed to detect a segments of the Meq gene in feather tip sample of chickens experimentally infected with MDV (Chang *et al.*, 2002) In chickens infected with Md5; a strain of oncogenes MDV, the Meq gene was consistently detected, whereas the L-meq gene, in which a 180 base pair (180 bp) sequence is inserted into the meq gene was detected in CVI988 infected chickens. Moreover the gene was mainly detected even in chickens co-infected with both Md5 and CVI988 (Upadhayay and Ewam, 2012; Okazaki *et al.*, 2009).

2.9.2.1.2. MULTIPLEX POLYMERASE CHAIN REACTION

Simultaneous detection of MDV 1, avian leukosis virus and reticulo-endotheliosis virus in tumour tissues of naturally infected chickens and turkeys has been developed using multiplex PCR (Caterina *et al.*, 2004). After a decade of employing the molecular diagnosis of those viruses, which replicated conventional virus isolation, the development of real time multiplexes PCR for the simultaneous detection of both viruses (Upadhayay and Ewam, 2012). The real time PCR for MDV and for CAV alone are more sensitive than the respective end-point PCRs. In addition, the multiplex real time shows a similar sensitivity when compared to the single real time PCR for each virus. The newly developed real time multiplex PCR is of important in terms of the diagnosis and detection of low copies of each virus, MDV and CAV in single and in multiple virus infections, and its applicability will be further evaluated (Zelege *et al.*, 2005).

2.9.2.1.3. QUANTITATIVE REAL TIME PCR

Quantitative real time PCR to quantify MDV genome copies have been described for simultaneous detection and quantitation of viral load in clinical samples or infected tissues (Walkden *et al.*, 2013). Since MDV 1 ubiquitous, its quantitation in suspected clinical samples will be of diagnostic value rather than more detection by PCR. The quantitative real time PCR can be also used to monitor the vaccines (Islam *et al.*, 2006). Witter and colleagues 1998 developed a useful strategy toward differential diagnosis of viral lymphomas in chickens based on quantitative real-time PCR (RT-qPCR). However, both procedures require fresh or frozen tissue (Cortes *et al.*, 2011). Although qPCR has been used in the diagnosis of MD in tumor imprints, feather pulp, or blood collected in FTA cards, most diagnostic laboratories routinely process affected tissues for microscopic examination to confirm diagnosis of avian tumors and, thus, there is a need to determine whether DNA extracted from formalin-fixed paraffin embedded (FFPE) and formalin-fixed (FF) tissues can be used for detection of these two important avian tumor viruses, namely MDV and REV. It is also recognized that fresh or frozen tissues are not always readily available, and shipping (Zelnik *et al.*, 2004).

2.9.2.2. LOOP MEDIATED ISOTHERMAL AMPLIFICATION TECHNIQUE

Loop mediated isothermal amplification technique is used for rapid detection of marek's disease virus Meq gene in feathers of affected birds has been developed lately. Loop mediated isothermal amplification technique test required 100 fold less copy number for detection of MDV compared to conventional PCR, and the detection time can be less than sixty minutes (Angamuthu *et al.*, 2012). The Loop mediated isothermal amplification technique utilizes three different sets of primers binding to six different sequences thus add more specificity, the reaction is carried out at isothermal conditions and the products can be visualized by the naked eye (Woźniakowski and Samorek-Salamonowicz, 2014a).

2.10. DISTRIBUTION

2.10.1. DISTRIBUTION OF MAREK'S DISEASE IN THE WORLD

Marek's disease virus has a worldwide distribution, occurring in all poultry producing areas. Marek's disease is responsible for great economic losses to the poultry industry worldwide. Sporadic outbreaks of MD have been reported recently throughout the world even in vaccinated flocks, including India (Morrow and Fehler, 2004).

The questionnaire survey which were used for the analysis of the incidence of MD in 55 countries revealed that only five countries, including Peru in Latin America, reported economic losses caused by MD since the 1990's (Dunn and Gimeno, 2013). Mexico, Columbia and Venezuela reported outbreaks in the early 2000's, while the USA, Canada, Brazil and Argentina did not report outbreaks or indicated that the outbreaks during the 1990's were under control (Morrow and Fehler, 2004). MD outbreaks were also reported in a few European countries including Russia with economic losses as well as in China (Atkins *et al.*, 2011). MD is only occasionally diagnosed in the Americas and the incidence is decreasing in most of South, Central and North America with the exceptions of Bolivia, Columbia, Peru, Uruguay and Venezuela (Laurent *et al.*, 2001). These responses contrast with the responses to the question if MD is considered to be a problem, with all Latin American countries answering that MD is a considered a problem by most responders. In the rest of the world, MD is occasionally diagnosed, with the exception of several countries in Central Africa and a few countries in East Europe (Morrow and Fehler, 2004). China, India and Australia provided mixed responses. A remarkable change is the situation in Russia, which reported in 2004 economic losses but the recent survey by Dunn indicated that the frequency is decreasing and that MD is only occasionally diagnosed (Dunn and Gimeno, 2013).

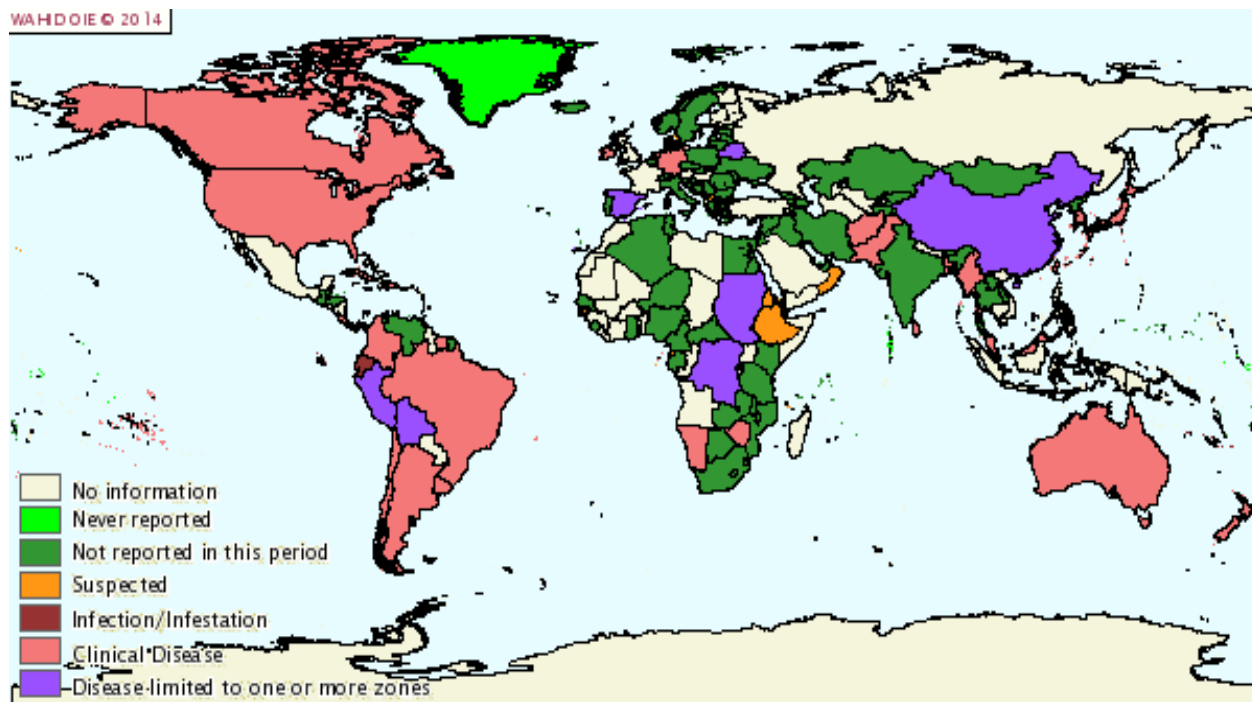


Figure 5: Worldwide map depicting distribution of MDV whereby evidence for presence has been submitted to the OIE (2014).

Distribution data was obtained from the World Organization for Animal Health (OIE) distributed through the World Animal Health Information Database (WAHID) interface and summarized above based on information and presence of disease.

2.10.2. DISTRIBUTION OF MAREK'S DISEASE VIRUS IN ETHIOPIA

In Ethiopia, Marek's disease was first diagnosed in 1983 and an incidence rate of 0.3% in industrial poultry farms was reported for the years 1983 – 1986 (Alamergot, 1987). Lobago and Woldemeskel (2004) conducted a study on an outbreak of Marek's disease in a commercial poultry farm containing 8500 chickens in central Ethiopia. The mortality rate was 46% for the first 14 weeks of the outbreak indicating its potential significance for modern chicken production in the country (Mazengia, 2012).

On the other hand Duguma *et al.*, (2005) investigated the prevalence, clinical and pathological manifestations and extent of mortality due to Marek's disease (MD) among indigenous chickens of Ethiopia reared under confined management at the Debre Zeit Agricultural Research Center, in central Ethiopia. The extent and effectiveness of Marek's disease (MD) vaccination is reducing the

incidence of mortality to natural MD challenge among 3 indigenous chickens ecotypes that was evaluated by Duguma *et al.*, (2006).

This study was conducted on indigenous chickens (Jarso, Tepi, Horro, and Fayoumi) chickens that were vaccinated against MD subcutaneously at neck region. Analyzed data by Duguma *et al.*, (2006) from daily records of mortality and survival of the chickens indicate that MD vaccination significantly increased the survival rate of Jarso, Horro and Fayoumi. The Tepi ecotype also showed improvement in survival rate due to MD vaccination but the improvement was not significant. This indicates the dramatic effect of MD vaccination on the survival of the indigenous chickens and it should be considered as an important management strategy while rearing them under confinement (Zelege *et al.*, 2005).

The results of the study made by Demeke *et al.*, (2017) indicated an overall significant difference between manifestation of clinical signs in acute (65.2 %) and chronic forms (34.8%), respectively. MD fatality rate was very high in this study where 97.9% diseased birds died. Serological prevalence study of MD using the Agar gel immuno-diffusion test (AGID) technique on 70 randomly sampled local chickens revealed an overall prevalence rate of 72.9 %. Information on circulating MD strains isolation in different production systems is also lacking. The studies summarized above indicate that MD is a serious problem for the poultry industry of Ethiopia and vaccination could be a vital instrument to combat against the threat. However, despite its growing importance of MD vaccine is being availed to the market by importation (Zelege *et al.*, 2005).

Study conducted by Berhan (2014) on isolation and molecular characterization of Marek's disease virus from clinically diseased Ethiopian chickens reared under different production system; the result revealed that all Ethiopian Marek's disease virus isolates were clustered under *Gallid Herpes virus* type 2. Currently because of the high demand of vaccine by the private and government poultry production farms in the country, the National Veterinary Institute is importing Marek's disease vaccine from India. Assessment of outbreaks in a country level and identification of the circulating virus type is very important to reach in a decision on the selection of safe and protective vaccine strain (Reta, 2009).

2.11. CONTROL AND PREVENTION OF MAREK'S DISEASE

2.11.1. VACCINATION

In 1967, the first isolation of MDV was successfully achieved and an enormous effort for vaccine-development was independently performed. In early 1970s, neonatal vaccination with live apathogenic MDV was found to prevent lymphoma formation, and MD was effectively controlled by vaccination. Currently, all three serotypes of MDV, serotype 1 (attenuated strains), (Rispen *et al.*, 1972) serotype 2 (non-pathogenic strain) and serotype 3 (herpes virus of turkey, HVT) are used as vaccines (Okazaki *et al.*, 1970; Witter *et al.*, 1970).

Live virus vaccines, used since 1970, remain the basis of disease control programs. These are usually administered to day-old chicks at hatching to provide protection against the natural challenge the chicks are exposed to early in life from the infected poultry house environment. With the introduction of *in ovo* immunisation methods, an increasing number of birds are vaccinated by this route. Marek's disease vaccines are highly effective, often achieving over 90% protection under commercial conditions (Davison and Nair, 2004). Vaccines available vary from country to country. In the United States of America (USA), strains of MDV belonging to all three serotypes have been licensed as vaccines (Vaccine strain: FC126 (HVT), SB-1, 301B/1, CVI988 clone C, CVI988/C/RB, CVI988 (Rispen), and R2/23 (Md 11/75) and serotype 3,2,2,1,1,1,1 respectively) (Witter, 1998a).

In many countries, HVT continues to be widely used as a monovalent product because it is inexpensive, available as cell-free and cell-associated forms and effective when the field exposure is not severe. The HVT and SB-1 strains comprised the first commercial bivalent vaccine based on the protective synergism demonstrated between serotypes 2 and 3 viruses. The CVI988 strain Rispen vaccines and modified versions are widely used in many countries and appear to be effective against some of the vv+MDV pathotypes (Venugopal *et al.*, 2001).

Although MD vaccines have been successful in controlling major losses from the disease, threat of vaccine failure has continued to cause concern. According to the reasons for these possible failures include the following:

- A. challenge with virulent viruses before the development of vaccinal immunity,
- B. interference with the development of immunity by the maternal antibodies,
- C. improper use of the vaccine, and
- D. the use of a non-protective vaccine strain.

Vaccinating alternate generations with different types of vaccines can reduce the effects of interfering passive antibodies. Early exposure to MDV can be significantly prevented by improved hygiene and biosecurity measures. Despite the success achieved by vaccines in controlling MD, the continuous evolution of MDV strains towards greater virulence leading to the emergence of vv and vv+ pathotypes of MDV is threatening to pose problems in the future (Upadhayay and Ewam, 2012). The development of more effective vaccines through recombinant DNA Laney technology, and the use of immune modulatory approaches to enhance the response to vaccines should improve vaccination strategies in the future (Kano *et al.*, 2009).

2.11.2. SELECTION FOR GENETIC RESISTANCE

The best way to prevent Marek's Disease is also to develop a family of birds that is naturally resistant against the virus. Acquire families of birds and use sires that are resistant to MD, because there is strong level of heritability for genetic resistance against MD (Warner *et al.*, 1987). Alternatively, following an outbreak use strong male survivors as these birds have shown a resistance to MD. Developing a family of birds that is genetically resistant to MD is by far the best way to cure the disease (Cheng *et al.*, 2008).

2.11.3. HYGIENE MEASURES

The use of vaccines should never be an excuse for poor management or lack of biosecurity measures. Particularly for commercial flocks, it is important to have good biosecurity to ensure that vaccinated chicks will develop immunity before they are subjected to a severe challenge of virus (Baigent and Davison, 2004). For example, chicks need to be reared separately so that they are free from the infected fluff and dust of older birds. Standard hygiene measures are also important, including a thorough clean-out and disinfection of sheds and equipment between batches of chicks with a disinfectant effective against viruses. Good nutrition and maintenance of freedom from other diseases and parasites are also very important. These practices will help maintain the flock's health and to ensure that the birds have optimum resistance against Marek's disease infection (Payne and Venugopal, 2000).

3. MATERIAL AND METHOD

3.1. DESCRIPTION OF STUDY AREA

The study was performed from February 2020 to November 2020 in four selected areas of Mid Rift Valley of Ethiopia, based on MD outbreak report. Samples were collected from Adama, Batu, Bishoftu, and Hawasa poultry farms which were purposively included in the present study since MD outbreaks were occurred during the study period. These areas were known as the main belt for poultry production in in Mid Rift Valley of Ethiopia (figure 5). Adama is a special zone of Oromia and is surrounded by East Shewa Zone. It is located at 8°32'N 39°16'E latitude and 8.54°N 39°.27°E longitude at an elevation of 1712 meters above sea level and 99km southeast of Addis Ababa. Batu town is located on the latitude 7°56'N 38°43'E and longitude 7.933°N 38.71°E at an elevation of 1643 meters above sea level. It is located on the road connecting Addis Ababa to Nairobi in the East Shewa Zone of the Oromia region state at distance of 168.4km. Bishoftu is a town located in the East Shewa Zone of the Oromia Region. It is located 8°45'N 38°59'E latitude and 8.750°N 38.983°E longitude at elevation of 1920 meters above sea level. It is located at 47.9km southeast of Addis Ababa. Hawassa is the city in the shore of lakes in the Great Rift Valley. It is located latitude 7°3'N 38°28'E and longitude 7.050°N 38.467°E and an elevation of 1708 meters above sea level and 273km south of Addis Ababa.



Figure 6: Map of Ethiopia showing the study areas where MD outbreak samples were collected from clinically diseased chickens (Source: own presentation).

3.2. STUDY POPULATION

The study populations were chickens that showed Marek's disease symptoms found in selected areas in Mid Rift Valley of Ethiopia. A total of 164 clinically sick chickens of all ages and breeds reared under backyard and commercialized cage production were examined in the study.

3.3. STUDY ANIMALS

The study animals were chickens from backyard/villages and commercial farms that had experienced outbreaks of Marek's disease.

3.4. STUDY DESIGN

The study employed a cross sectional study design following the report of Marek's disease outbreak for MDV detection and strain investigation from backyard (non-vaccinated) and commercial chickens located in the study area by collecting feather samples from February 2020 to November 2020. The samples were collected purposively twice from the local markets located in the study area.

3.5. SAMPLE SIZE DETERMINATION

The sample size will be calculated based on the prevalence of 72.14% as previously reported by (Zelege *et al.*, 2005a) using 95% confidence level and 5% margin error. The previously recommended formula (Thrusfield, 2005) was used to determine the required sample size as indicated below.

$$n = \frac{Z^2 P_{\text{exp}} (1 - P_{\text{exp}})}{d^2}$$

Where;

n= Sample size

z= Statistic for a level of confidence

d²= Required absolute precision

P_{exp}= Expected prevalence

Therefore, at the 95% confidence level, the sample size is:

$$\begin{aligned}n &= \frac{1.96^2 P_{exp} (1-P_{exp})}{d^2} \\n &= \frac{1.96^2 \times 0.7214 (1-0.7214)}{(0.05)^2} \\n &= \frac{3.8416 \times 0.7214 (0.2785)}{0.0025} \\n &= 308\end{aligned}$$

Hence, a total of 308 chickens were required to be sampled (feather) for determination of the molecular prevalence and strain identification of MDV. However, to improve the accuracy of the study, 164 chickens were randomly selected based on clinical signs and symptoms of MDV in from village, commercial, and poultry markets in the study area.

3.6. QUESTIONNAIRE SURVEY

Questionnaire survey was carried out in poultry farms where MD outbreak occurred. A total of 38 respondents were interviewed using semi-structured questions for epidemiological data regarding total chickens, number of affected chickens, number of chickens reported dead due to MD, farming system, age, breed, sex, clinical findings, vaccination history from all poultry farms. Clinical examination result and post mortem findings were recorded in the prepared format (Appendix I).

3.7. SAMPLE COLLECTION AND TRANSPORTATION

During sample collection history of the farm and basic information about the disease was recorded to know mortality and morbidity rate in respect of vaccination history. Suspected clinical chicken's samples were collected carefully from individual cases. A total of 164 clinically sick chickens (46 from Adama, 10 from Batu, 66 from Bishoftu and 42 from Hawasa) of all ages and breeds reared under back yard and commercialized cage production were examined in the study. Outbreak investigation was conducted in a total of 164 chickens, where 113 female layer chickens (41 from Bishoftu, 36 from Adama, 29 from Hawasa and 7 from Batu) and 51 male broiler chickens (19 from Bishoftu, 11 from Adama, 17 from Hawasa and 4 from Batu) in 15 poultry farms. From 164 clinically sick chicken 82 pooled of feather were collected. Samples were placed in tube contains VTM; labeled and transported using cold-chain to the diagnostic laboratory of the NAHDIC. In the laboratory samples were kept at -80°C in order to maintain the viability of virus, until laboratory analysis is carried out.

3.8. LABORATORY PROCESS

3.8.1. SAMPLE PREPARATION

The Feather follicles that were collected from suspected chickens were held by sterile forceps, and then cut with a sterile scissors 1 cm (4 pieces) from the tip of feather at the terminal position. Feather from the same outbreak farm were pooled and processed inside the Biosafety cabinet level II as one sample to increase the chance of virus DNA isolation. Samples were lysed by adding chemical reagent, with different temperature and time of incubation depending on the feather size. 20 µL tissue Samples were used per purification by homogenizing in 400 µL buffers, then 200 µL supernatant were used by incubation at room temperature for 30 minutes. Centrifuges at 16000 rpm for 15 minutes, then the supernatant were used for viral nucleic acid extraction.

3.8.2. VIRAL DNA EXTRACTION

For DNA extraction 180µl buffers ATL were pipetted and 20µl proteinase K were added to the tubes contains supernatant were vortexed and centrifuged then incubated at 56 °c for 20 hours. 200µl buffer Al were added and mixed for 15 seconds and the mixture incubated for 10 minutes at 70 °c and centrifuged briefly at 8000 rpm and 40⁰c. After the suspension were centrifuged briefly with centrifuge tubes and 200 µl of molecular grade Ethanol (96-100%) were added and mixed for 15 seconds. This mixture was applied into QIAamp spin column to precipitate.

The QIAamp spin column were placed in a clean collection tube and centrifuged for 1 minute at 8000 rpm; then the collection tube with filtrate were discarded. The QIAamp spin column were opened carefully 500 µl buffers AW1 were added and placed in new collection tubes, then centrifuged for 1 minute at 800rpm. The collection tubes with filtrate were discarded. The QIAamp spin column were opened carefully 500 µl AW2 buffers were added and placed in new collection tube, then centrifuged for 3 minute at 20000 rpm. The collection tubes with filtrate were discarded. The QIAamp spin column were placed in sterile reaction vessel and 200 µl AE buffers were added then incubated for 1 minute at room temperature, the centrifuged for 1 minute at 8000 rpm and the filtrate were retained at 4⁰c for 48 hours. Finally, samples were centrifuged for 1 minute at maximum speed 35000 rpm. The Eppendorf tube containing the purified DNA was used for Quantitative Real-Time PCR.

3.8.3. REAL-TIME QUANTITATIVE POLYMERASE CHAIN REACTION (RT-qPCR)

RT-qPCR was done according to manufacturer's instructions, after MDV DNA extraction from feather follicle by using QIAamp DNA Mini Kit. By using an Applied Biosystems 7500 fast real-time PCR thermo cycler, the following master mix reagents per 20 μ l reaction: 10 μ l platinum PCR mix, 10 μ l of PCR buffer (10x), 0.6 μ l of forward MDV-1 primer (5'-GTCTGACTTCGAGACGGAAAA-3') and 0.6 μ l reverse primer (MDV1OP R: 5'-CAGAAGAGGGAATGGGGAGT-3'), 0.8 μ l (dNTPs), 1U Taq DNA polymerase, 0.8 μ l probe (5'-CGTCTTACCGAGGATCCCGAACAGG-3'), 1.25 μ l of MgCl₂ and 1 μ l of BSA 5mg/ml, 2 μ l of DNase and RNase free H₂O water mix were used (QIAGEN). All reagents were mixed in a single reaction tube (master mix) to ensure the reagents were distributed homogeneously. Depending on the number of samples to be tested, aliquots of 17 μ l of the prepared reagents were distributed into the applied biosystem plate by adding 3 μ L templates DNA. Finally, The Real Time PCR (RT-qPCR) were carried out in a final reaction volume of 20 μ L using a 200 μ L capacity thin wall PCR tube. Known positive MDV antigen and DNase and RNase free water were used as positive and negative controls, respectively. To avoid evaporation, the plates were sealed with a sealer and placed in a thermal cycler machine that was connected to the computer with its own software (Applied Biosystems Sequence Detection Software Version 2.3), and the program was adjusted accordingly for each specific reaction. The RT-qPCR protocol was performed with an initial denaturation at 95°C for 15min, followed by 45 cycles of denaturation at 95°C, annealing at 60°C and extension at 72°C each for 30sec, and final extension at 72°C for 5min. At the extension phase of each cycle, the fluorescent dye FAM, Cy5 and TEX signals were measured, and the threshold cycle (Ct.) for each sample was determined. Samples with a Ct. Value of greater than 42 were considered negative for Meq genes based on RT-qPCR (OIE, 2014).

3.9. DATA ANALYSIS

The data were summarized based on study participant's respond and laboratory investigation. The encoded data was stored on the Microsoft Excel 2016 spreadsheet and transferred for statistical analysis to SPSS Version 20. The outcome was therefore presented using descriptive statistics such as percentage and P-value.

3.10. Ethics approval

Sampling from animals were carried out according to the experimental practice and standards approved by the University Ethical Review Board (UERD) from Research Affairs Dean Office, Adama Science and Technology University (ASTU), with the verification number RECSOANS/BIO/03/2020 (Annex VI), that is in accordance with the international guidelines for animal welfare.

4. RESULTS

4.1. QUESTIONNAIRE SURVEY RESULT

Based on questionnaire survey result, (18.42%) (7/38) of the respondent clean the chickens room daily while (81.57%) (31/38) do not; (65.78%) (25/38) of the respondent vaccinated their chickens for MDV while (34.21) (13/38) do not; (81.57%) (31/38) of the respondents have knowledge about causes, transmission, prevention and control of MD while (13.16%) (5/38) do not; from observed poultry farm 11 (73.3%) have biosecurity while 4 (26.66%) do not have. From a total of 164 chickens 146 (89.02%) were sick 97 (66.43%) were from poultry farm while 49 (33.56%) were from backyard and 18 (10.97%) were at risk 7 (38.88%) from poultry farm while 11 (61.11%) were from backyard. 68 (41.46%) chickens from 3-18 weeks of age were more susceptible than other with less than < 3 weeks of age {23 (14.02%)} and greater than >18 weeks of age {28 (17.07%)} (Annex I) (Table 2).

Table 2: Summary of questionnaire survey

Study area	Production type	Age (weeks)	No. Sick chickens	No. risk chickens	Sex	Vaccination history
Adama	Broilers and layers	1-18	42	4	32 female	37 vaccinated
					14 male	9 unvaccinated
Batu	Broilers and layers	1-16	8	2	7 female	10
					3 male	Unvaccinated
Bishoftu	Broilers and layers	1-24	60	6	43 female	51 vaccinated
					17 male	15 unvaccinated
Hawassa	Broilers and layers	1-28	36	6	31 female	29 vaccinated
					17 male	13 unvaccinated
Total			146	18	113 female	127 vaccinated
					51 male	37 unvaccinated

The current molecular findings indicated an overall prevalence of 16.23% MD in backyard chickens, whereas 14.13% overall prevalence in poultry farms in the study area. There was a statistically significant difference ($p < 0.05$) among the production systems (Table 3).

Table 3: Prevalence of MD in the production system (backyard and commercial) in the Mid-Rift Valley of Ethiopia based on feather follicle samples.

Study area	No. of chickens examined		No. of positive		Prevalence %	
	Backyard	Commercial	Backyard	Commercial	Backyard	Commercial
Adama	7	39	0	2	0	5.13
Batu	3	7	0	0	0	0
Bishoftu	14	52	1	3	7.14	5.77
Hawasa	11	31	1	1	9.09	3.23
Total	35	129	2	6	16.23	14.13

Based on the geographical location of the study area, the overall prevalence of MD in the Mid Rift Valley area was found to be 9.76 % (Table 4) where the highest was at Bishoftu (12.12%) and followed by Hawasa (9.52%). There was a statistically significant difference ($p < 0.05$) among the study geographical locations.

Table 4: Prevalence of MDV based on the geographical location of the study area

Geographical location	No. Examined	No. Positive	Prevalence (%)
Adama	23	2	8.69
Batu	5	0	0
Bishoftu	33	4	12.12
Hawasa	21	2	9.52
Total	82	8	9.76

4.2. CLINICAL EXAMINATION RESULT

From 164 clinically examined chickens, 18 chickens (10.76%) developed paralysis of leg, neck and wing (figure 7). 146 (89.02%) developed other clinical signs such as depression, loss of appetite, weight loss, brown eye color, loss of feather, difficulty in breathing. The clinical signs were mostly appeared in chickens with 3 to 16 weeks of age. Out of 18 paralyzed chickens, 13 (73.68%) occurred in unvaccinated whereas 5 (26.32%) occurred in vaccinated chickens (Table 5). This implied that vaccination has been reduced the occurrence of paralysis.



Figure: 7. clinically diseased chicken suspected of MDV infection (Source: picture taken by Temam)

Table 5: Clinically diseased chickens examined for MD

Study area	No. examined	Type of organ	No. paralyzed	Vaccination history
		Feather		With HVT strain
Adama	46	23 pools	1	Vaccinated (HVT Fc126)
			3	Unvaccinated
Batu	10	5 pools	1	Vaccinated (HVT Fc126)
			2	Unvaccinated
Bishoftu	66	33 pools	2	Vaccinated(HVT Fc126)
			5	Unvaccinated
Hawasa	42	21 pools	1	Vaccinated(HVT Fc126)
			4	Unvaccinated
Total	164	82 pools	5/13	

MDV is very high in non- vaccinated chickens when compared with vaccinated. Only five chickens show paralysis from vaccinated while 14 chickens show paralysis from unvaccinated.

The present finding revealed that the prevalence of MD was not similar in old and young chickens. 12% of Adult chickens and 10.62% female chickens were exposed to the Marek's disease virus out of the total population in the study area with a statistically significant difference by age ($p < 0.05$) (Table 6).

Table 6: Prevalence of MD in chickens by Sex and Age

Categories	No. Examined	No. positive	Prevalence (%)
Sex			
Female	113	12	10.62
Male	51	4	7.84
Age			
Young	45	2	4.44
Adult	100	12	12
Old	19	2	10.53

4.3. REAL TIME QUANTITATIVE POLYMERASE CHAIN REACTION (RT-qPCR)

The current findings indicate that out of 164 chickens with 82 pooled in five feather follicle samples tested by RT-qPCR for MDV, 8 (9.76%) were identified as positive for MDV (Table 7), indicated with its Ct. value when read from Applied Biosystems 7500 fast real-time PCR thermocycler (Figure 8 and Figure 9). Out of the 82 pooled samples tested by RT-PCR for Meq gene assay the recorded highest prevalence was found at Bishoftu (4.88%), followed by Adama and Hawasa (2.44%), and the least prevalence was recorded at Batu (0%).

Table 7: Prevalence of Marek's disease by RT-qPCR tests with Ct. Values for Meq gene in the Mid Rift Valley of Ethiopia.

Sample ID	Sample type	Location sample	of live or died animal	Ct Values
A ₇	Feathers	Adama	Live	34.21
A ₁₂	Feather	Adama	Live	31.09
BS ₁	Feather	Bishoftu	Live	33.59
BS ₅	Feather	Bishoftu	Live	32.96
BS ₇	Feather	Bishoftu	Live	33.99
BS ₁₇	Feather	Bishoftu	Live	34.08
H ₁₉	Feather	Hawasa	Live	31.94
H ₂₁	Feather	Hawasa	Live	32.90
Positive control	MDV Antigen			21.64
Negative control	DNA and RNase free water			>35
Mean Ct. value for M-gene				29.42

Real-Time PCR test was valid because the FAM-, Cy5- and TXR-curves of the Negative Control are negative, the HEX-curve of the Negative Control is positive and the FAM-, Cy5- and TXR-curves of the Positive Control are positive. For a valid test the FAM-, Cy5- and TXR-Ct-values of the Positive Control has to be > 15 and ≤ 35 and the HEX-Ct-value of the Negative Control has to be ≤ 40 . The samples which were negative and positive for virus isolation were also showed in RT-qPCR (Figure 8) (Table 8).

Table 8: MDV positive result of RT-qPCR by fluorescent probe

Target	Channel	Study area and Signal			
		Adama	Batu	Bishoftu	Hawasa
Internal control	HEX	Positive/ Negative	Positive/ negative	Positive/ Negative	Positive/ negative
MDV field strain	TXR	1 positive	—	1 positive	—
MDV HVT Fc126 vaccinated	Cy5	1 positive	—	2 positive	2 positive
double infection with MDV field and HVT Fc126 vaccine strain	FAM, Cy5, and TXR			1 positive	—
Total		2	0	4	2

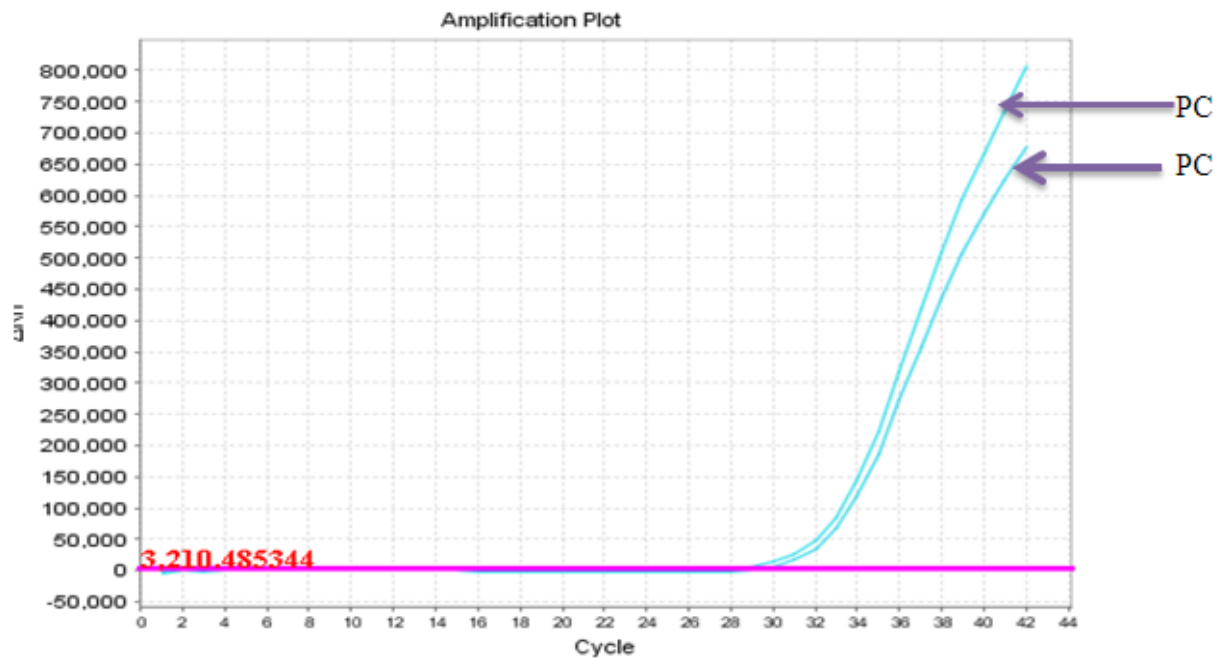


Figure 8: MDV positive control

A samples detected positive for MDV field strain has FAM- and TXR-curves positive ($Ct \leq 42$), independent of the Cy5- and HEX-curve one from Adama and one from Bishoftu. A samples detected positive for MDV vaccine strain HVT Fc126 has FAM- and Cy5-curves positive ($Ct \leq 42$), independent of the TXR- and HEX-curve one from Adama, two from Bishoftu and two from Hawasa. A potential double infection with MDV field strain and MDV HVT Fc126 vaccine strain was FAM-, Cy5- and TXR-curves positive ($Ct \leq 42$), independent of the HEX-curve one from Bishoftu was showed fluorescent probe of RT-qPCR. Graph of MDV positive result is presented in (figure 9 and 10).

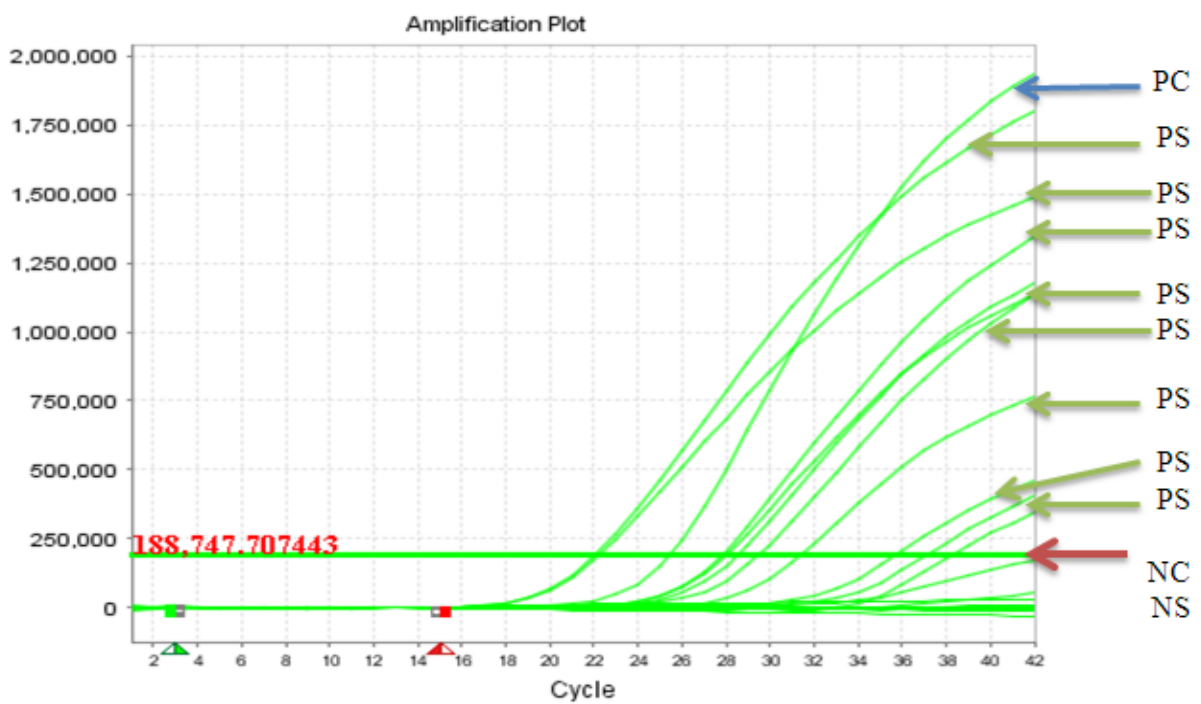
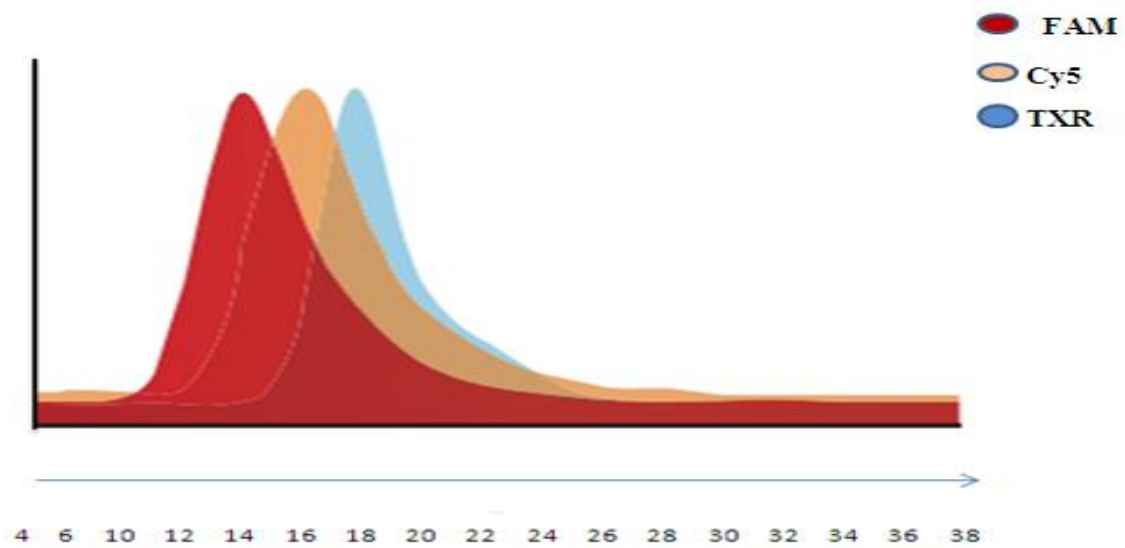


Figure 9: Amplification plot results of RT-qPCR with their Ct. Value positive sample (PS), positive control (PC), negative sample (NS), and negative control (NC).

5. DISCUSSION

The present study on MD from both vaccinated and unvaccinated chickens of backyard and poultry farm by using RT-qPCR revealed an overall prevalence of 9.76% in the study area ranging from 0% at Batu to 4.89% at Bishoftu, even though, the distribution of the disease differ among the study areas in Mid Rift Valley using RT- qPCR analysis with statistically significance difference ($p < 0.05$). PCR study conducted by Mirtnehe (2015) in six selected districts (Adama, Addis Ababa, Dukem, Akaki, Modjo, and Bishoftu) in rift valley area revealed an overall prevalence of 93.33% which is greater than the current finding and this could be due to the small area coverage. The negative results from the qPCR test in Bishoftu seem strange but a molecular and serological study conducted for MDV from Egypt in non-vaccinated backyard chickens also showed negative result on PCR but positive reaction on Haemagglutination inhibition (HI) test (Fatma *et al.*, 2018). They conducted that the serological finding may imply a circulation of MDV antibody on the native chickens in the area while molecularly; no virus was detected since there was no occurrence of active viral infection.

The result of the present study (9.76%) was greater than the finding of other research done by serological detection method in Jarso, Konso, Tepi, Tilili, and Horro with overall seroprevalence of 7.09% (Duguma, 2005). In other serological study conducted in selected area in rift valley an overall sero-prevalence of 5.6% was reported (Lobago and Woldemeskel, 2004) and 11.6% (Yami, 2005) from which one can discern out that result under the present study, molecular tools, out weight the result of the former studies by serological (ELISA) method. The present higher proportion (42.86%) of qPCR positive result for MDV in chickens from Adama district indicate chickens have been previously infected by the non-virulent strain or survived outbreak of the virulent NDV strains (Duguma, 2005).

Moreover, the detected high prevalence of MD in this study may mean that natural infection could have occurred since all the chickens were non-vaccinated. However, clinical signs of MD were not observed on the examined chickens during sample collection. Furthermore, the higher prevalence recorded in Bishoftu cannot be attributed to more chickens had been sampled (70 chickens sampled) compared to other study district (70 chickens sampled), because as the number of sample size increase the probability of having higher prevalence's also increase. In addition, according to the report by Kenenet (2017) and Berhan (2016) low altitudes do have higher sero-prevalence than

the high altitude in their study and they were investigated as there were few chickens in the highland area. Hence, chickens population number is a factor for the transmission of the disease in their study. The present study also revealed significant variation with almost closer agro-ecology between Adama and Bishoftu.

The difference in the prevalence between adult, young and old age was statistically significance ($p < 0.05$), which agrees with the finding of Berhan (2014) who state that the young had a significantly lower MD prevalence than the adult. Furthermore the result of the current study corroborates, with the finding of Kenenet (2017) from Kombolcha and Mekele of Ethiopia with sero-prevalence of 17.3% and 29.7% for young and adult, respectively. They hypothesized that, more frequent exposure of adult birds to field virus, which might have survived the disease at an earlier age.

The present study revealed a higher prevalence rate of MD among the male 16.1 (35/164) compared to female chickens 14.565 (47/164) with statistically insignificance difference ($p > 0.05$). This finding corroborates with the study reported by Duguma *et al.*, (2005) in central Ethiopia where a higher sero-prevalence rate among males (31.09%) than female (68.90%) was observed. In contrary to finding Mirtneh (2015) reported slightly higher sero-prevalence among females (32.63) chickens when compared with male (31.63%) chickens in Ethiopia. The higher prevalence of the disease observed in the present study could be due to higher number of male chickens involved by chance in the study area during sampling than females.

From the questionnaire interview, almost none of the chicken's owner had ever vaccinated their chickens (65.78%). In this study 92.11% of the respondents indicated that the decreased flock size was due to disease. This finding is in agreement with Duguma (2005) and Mirtneh (2015) who reported 7.09% and 9.6% of the respondents indicated their flock size decrease due to disease. These indicated that households had lost their chickens, possibly due to mainly incidence of disease in their flocks, among other factors. The majority of the respondents 92.11% indicated MD is increase the mortality in chickens of backyard and poultry farm. This was corroborated by PCR test positivity during the sampling time.

The current study showed an overall molecular prevalence of 9.76 % using RT-PCR, indicating MDV is an endemic disease. In Adama, Batu, Bishoftu and Hawasa we

detected a prevalence of 2.44%, 0%, 4.88%, and 2.44%, respectively. The MDV distribution among study areas varies having a statistically significant ($P < 0.05$) difference among the study districts. A 9.76% prevalence of MD in Mid-Rift Valley of Ethiopia is suggested by current molecular findings.

According to Blaha (1989), the difference in rate and duration of mortality in a flock and the age at which an outbreak of MD occurs are influenced by a number of factors. These are related to either the infective agent (viral strain, dosage and route of exposure), host factors (genetic constitution, age and sex), or environmental factors (defense-decreasing stressors). According to this author the complex set of factors for pathogenesis, and then the incubation period, character and extent of lesions and symptoms, the course of the disease, morbidity and mortality depend mainly on the above three set of factors. In addition to viral and/or chickens factors for higher susceptibility indicated previously above, confining and stocking of local chickens without MD vaccination might have contributed to the high mortality rate.

This is in agreement with the report of Palya (1991) and Blaha (1989) who have indicated high morbidity and mortality (that ranges from 20-70 %) of MD in intensive production system in non-vaccinated chickens. The observation of high mortality rate in this study also agrees with the findings of (Lobago and Woldemeskel, 2004) in that mortality as high as 46 % have been recorded in intensively managed non-vaccinated exotic chickens in central Ethiopia. However, it differs from the report of (Lobago *et al.*, 2003) who has observed a prevalence rate of 9.23 % and mortality rate of 5.51 % as well as the current finding prevalence rate 3.04% and mortality rate 0.61% of MD in vaccinated chickens.

Probably this variation is attributed to the MD vaccination of their study flocks and also genetic variation of the birds. In this line Palya (1991) and Blaha (1989) have indicated that MD vaccination has brought a difference to commercial poultry farms is successfully controlling MD, and hence its mortality incidence has dramatically dropped from ranges as high as 70 % in non-vaccinated to less than 5 % in vaccinated. According to (Palya, 1991) tumor formation in various organs is the characteristics of MD virus sero-type 1. During current study MD attack at 14 and 15 weeks of age was higher than the subsequent age groups. The occurrence of high magnitude of acute form of clinical signs and also visceral forms during autopsy with varying degree of

mortality rate among age groups were in agreement with the reports of (Lobago and Weldemeskel, 2004; Frasem and Mays, 1986; OIE, 1990). According to (Venugopal, 2005) there was also a report that acute and/or visceral forms of MD, with recent increasing evolution of virulent viral strain, have been frequently observed currently. Antibody prevalence to MD in this study is very high, 72.9 %. According to (OIE, 1990) AGID tests are employed most commonly to detect antibody and the presence of antibodies in chickens above 4 weeks of age is an indication of MD infection. The variation in magnitude of signs, lesions and mortality rates among chicken ecotypes despite nearly equal likelihood to MD infection might be related to genetic variation of the chickens. In this line based on PCR study on the DNA, (Tadelle, 2003) indicated the presence of genetic variation (distance) among these five chicken ecotypes of Ethiopia.

6. CONCLUSION AND RECOMMENDATIONS

Marek's disease is one of the major viral diseases of chickens in mid Rift Valley of Ethiopia. It is a highly contagious viral infection that predominantly affects chickens. The high morbidity and mortality of the disease can seriously threaten the poultry industry. The disease mainly affects three to six months of age chickens. Unvaccinated flocks are severely affected by the disease as compared to vaccinated ones but, the vaccine also couldn't completely stop the morbidity of the disease.

Based on the findings of this study the following points are recommended:

- ❖ Further study on molecular investigation of chickens Marek's disease virus in all regions of the country as well as studies should be conducted to be acquainted with the distribution of serotypes and pathotypes of Marek's diseases virus circulating in the country.
- ❖ Detailed study should be conducted on field effectiveness of the trial vaccine and targeting different breeds, geographical areas on back yard and commercial poultry farms.
- ❖ Commercial poultry farms should implement regular vaccination program, thereby reducing the impacts of MD.
- ❖ Development and production of effective vaccines of Marek's disease should be facilitated in the country which could protect the chickens from the circulating virus type.

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8. APPENDICES

Appendix I

Questionnaire format for survey

Part I: General Information

Date__

Region _____, Zone _____, District_____ City/village_____

Name of the respondent_____

Geographical positioning system data collection in selected mid Rift Valley areas.

Location	North (latitude)	East (longitude)	Altitude

Part II: Information related to the research purpose

1. How long have you been working with chicken keeping? __
2. How many which breeds and types of chicken do you have currently?

Chicken Type	Number of chickens by breed		
	Exotic	Hybrid	Local
Layer			
Pullets			
Chickens			
Cockerels			
Cocks			

3. Objectives of the farm? Egg production, meat production, breeding stock, mixed

4. Type of production system? Scavenging, semi-intensive, extensive
5. Level of biosecurity system? Low, moderate, high
6. Where do your chickens spend the day time (Housed/Scavenging)?
7. How far do your chickens move for scavenging and watering? ____
8. Is there any interaction of the chicken with other species of wild birds? Yes/No
9. Did you encounter a health problem in your chicken/farm? _____
10. Which disease causing high losses in your chicken/farm?
11. What is the major health problem affecting your chicken?
12. In which types of diseases and chicken mortality is more serious? _
13. At what season do you frequently encounter the disease problems?
14. Where and when the chicken is bought for production purposes? Do you use DOC?
15. In which types of diseases and chicken mortality is more serious? _
16. At what season do you frequently encounter the disease problems?
17. Do you vaccinate your chicken for these major diseases in your area? (Yes/No). If yes, which one? Source of vaccine?

Name of Major diseases	Affect ed Age	Affect ed Sex	Affect ed Breed	Affect ing Season	No of Sick	Clinic al sign	No of Dead

Way of transportation____ Carry by hand, by cart, by car

Did you clean chicken's room daily? YES/ NO

Did you encounter ND outbreaks? YES/ NO

Season of MD outbreaks?, Form short rainy season, long rainy season, dry cool season, throughout of the year.

The chickens get vaccination for MDV? If yes, which type of vaccine is used? ____

Do you Knowledge about cause, transmission, prevention, and control of MD? Yes/no

Age of poultry at MD is more pronounced? Starter, Layers, Broilers, Mixed, Not respond.

Breed at MD more pronounced? Local, Exotic, Cross

Did you observe outbreak (< 10 km distance from the market)? Yes/No

N. What do you think about disposing of dead birds and other materials that facilitate the occurrence of major diseases?

Appendix II

Supplementary Table 1: Questionnaire survey results on associated risk factors for ND in the study area.

Category of variable	N=	%
	38	
Way of transportation		
Carry by hand	23	60.52
By cart	15	39.47
Did you clean the chicken's room daily?		
Yes	7	18.42
No	31	81.57
Did you encounter MD outbreaks?		
Yes	38	100
No	0	0
Did you vaccinate your chicken for MDV?		
Yes	25	65.78
No	13	34.21
Season of MD outbreaks		
A short rainy season	18	46.15
Long rainy season	9	23.68
Dry cool season	11	28.94

Throughout year	0	0
Do you have Knowledge about causes, transmission, prevention and control of MD		
Yes	31	81.57
No	5	13.16
Not respond	2	5.26
Age of chickens at MD more pronounced		
From 2-4 week	0	0
From 5-10 weeks	6	15.78
From 11-18 weeks	27	71.05
Above 19 weeks	5	13.15
Breed at MD more pronounced		
Local	18	47.36
Exotic	15	39.47
Cross	5	13.15
Do you have biosecurity system?		
Yes	25	65.78
No	13	34.21
Did you observe out-break (< 10 km distance from market)		
Yes	19	50
No	19	50

Appendix III

DNA extraction

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.

Pipette 180 µl in this buffer ATL and 20 µl proteinase K and incubate at 56 °C for 20 hours. Vortex repeatedly and centrifuge briefly

Add 200 µl buffer A1 mix for 15 seconds and incubate the mixture for 10 minutes at 70 °C.

Centrifuge briefly

Centrifuge the suspension briefly with centrifuge tubes and add 200 µl of molecular grade Ethanol (96-100%) and mix for 15 seconds

Apply this mixture to precipitate in to a QIAamp spin column

The column is then centrifuge for 1 minute at 6000g(8000 rpm)

Place the QIAamp spin column in a clean collection tube. Discard the collection tube with filtrate.

Carefully open to the QIAamp spin column and add 500 µl buffer AW1.

The closed column is then centrifuge for 1 minute at 6000g (8000 rpm).

Place QIAamp spin column in a new collection tube and discard the filtrate

Carefully open to the QIAamp spin column and add 500 µl buffer AW2.close the tube and centrifuge for 3 minutes 20000g or 14,000 rpm.

Place QIAamp spin column in a sterile reaction vessel and discard the collection tube with filtrate.

Pipette 200 µl of buffer AE in the QIAamp spin column. Perform a 1 minute incubation at room temperature

Centrifuge for 1 minute at 6000g or 8000rpm and retain the filtrate at 4 °C for 48 hours at the latest. Prolonged storage at -20°C

Appendix IV

PCR Mix (Master Mix.) for MDV-PCR

<u>Reagents</u>	<u>Volume of (µl) final concentration</u>
Platinum PCR mix	10
Primer forward MDV-1F	0.6
Primer reverse MDV-1R	0.6
Probe MDV-1	0.8
BSA 5mg.ml-1	1

H2O	2
Sample DNA	5
Total volume (µl)	20

Appendix V

Real time PCR thermal cycle program

Target (°C)	Hold (hh:mm:ss)
Activation/denaturation	
1 cycle 95°C	00:15:00
45 amplification cycle	
95 °C	00:00:15
60 °C	00:00:15
72 °C	00:00:10
Cool	
1 cycle 40°C	00:05:00

Appendix VI

Reagents used

180 µl of buffer ATL
 20 µl of proteinase K
 200 µl buffer Al
 Ethanol (96-100%)
 500 µl of buffer AW2
 200 µl of buffer AE

Annex IV: Research Ethical Approval Letter

ADAMA SCIENCE AND TECHNOLOGY UNIVERSITY
RESEARCH AFFAIRS DEAN OFFICE
UNIVERSITY ETHICAL REVIEW BOARD (UERB)

Phone: 1888

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CERTIFICATE OF APPROVAL

To: Temam Geletu Nagesso

Department: applied Biology

Program: MSc

Study Title: molecular investigation of Marek's disease virus in chickens in selected area of mid rift valley of Ethiopia

Approval date: January 10, 2020

Certificate Ref. No: RECSOANS/BIO/03/2020

Based on the request, the University Ethical Review Board (UERB) members reviewed the proposal taking into consideration the following key points:

- 1) Whether the intended research project have any risk to the research team, to the participants, data collectors, the data to be collected, the institutes involved in the research, project partners, funders involved, viz.
- 2) How, where, and by whom participants will be identified, approached, and recruited.
- 3) Type of people recruited.
- 4) The consequences of the research, risk of the data, how will it be protected and addressed
- 5) Consideration of anonymity and confidentiality

In light of the above key points, UERB members identified no any risk to any of the participants, institutions, data collectors and viz. Therefore, UERB recommend the implementation of the project as per the approved proposal.

Finally, I congratulate you for securing this Ethical Approval Certificate to precede your project.

Wishing you all the best!


Alemu Disassa Muleta
Research Affairs D.
Chairperson of University Ethical Review Board

