Thesis Ref. No.___

ISOLATION AND MOLECULAR CHARACTERIZATION OF MAREK'S DISEASE VIRUS ISOLATES CIRCULATING IN CENTRAL ETHIOPIAN CHICKENS

MSc. THESIS

By

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June, 2014

Bishoftu, Ethiopia

ISOLATION AND MOLECULAR CHARACTERIZATION OF MAREK'S DISEASE VIRUS ISOLATES CIRCULATING IN CENTRAL ETHIOPIAN CHICKENS

A Thesis submitted to the College of Veterinary Medicine and Agriculture of Addis Ababa University in the partial fulfilment of the requirements for the degree of Master of Science in Veterinary Microbiology

BY
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JUNE, 2014
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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: Berhan Demeke entitled " Isolation and molecular characterization of marek's disease virus isolates circulating in central Ethiopian chickens" and recommend that it be accepted as fulfilling the thesis requirement for the degree of: Masters of Science in Veterinary microbiology.

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Date of Submission: 09/06/2014

ACKNOWLEDGEMENTS

First of all the author expresses her profound thanks to her major advisor, Dr. Biruk Tesfaye, for his constructive comments and valuable suggestions, which helped her work to be this level. Profound thanks are also due to her co-advisors Dr. Shiferaw Jenberie and Dr. Esayas Gelaye, for her constructive comments and valuable suggestions, which have improved the paper. Grateful acknowledgement and appreciation is extended further to Dr. Esayas Gelaye for his unreserved help in facilitating the sequencing and phylogenetic analysis of the samples, interpreting the results and supplying relevant materials which helped her from the inception to the completion of this work.

The author thankfully appreciate the dedication of National Veterinary Institute (NVI) and its management in building the capacity of its staff through which she enjoyed study leave permit during her study periods. She owes also debt of gratitude to NVI for the financial support for her study and research together with the permit to use its laboratory facilities.

The author extends special appreciation to her parents W/r Anchinayahilush Mekonen and Ato Demeke Meshesha, her sisters and brother and their families for their unreserved support and encouragement during her study. Special thanks go to NVI colleagues Albachewu Belay, Hawa Mohamod, Ejgayhu Besha, Kider Hirepo, Bogale Gosaye, Ayele Teshome, Misrke Taddsse, and Selam Negatu who contributed for the accomplishment of this work.

Finally, the author would like to express her sincere and heartfelt thanks to her husband Workineh Abebe and her gifts from the almighty God, Arsema and Michael, for their love, encouragement, inspiration and understanding during the whole period of her study.

DEDICATION

This thesis/dissertation is dedicated to God the Father, God the Son, and God the Holy Spirit.

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ABBREVIATIONS

CEF	Chicken Embryo Fibroblast
CPE	Cytopathic effect
dpi	days post-infection
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphates
FBS	Foetal bovine serum
FFE	Feather follicle epithelium
GaHV-2	Gallid Herpes virus Type 2
GMEM	Glasgow minimum essential medium
HVT	Herpes Virus of Turkey
MD	Marek's Disease
MDV	Marek's Disease Virus
mMDV	mild Marek's disease virus
NCBI	National Centre for Biotechnology Information
NVI	National Veterinary Institute
OIE	Office International des Epizooties (World Animal Health Organization)
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
RPM	Rotation per minute
SPF	Specific pathogen free
TCR	T cell receptor
TPB	Tryptose phosphate broth
vMDV	virulent Marek's disease virus
vvMDV	very virulent Marek's disease virus
vv+MDV	very virulent plus Marek's disease virus
UL	Unique long
US	Unique short
W/V	Weight per volume

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ABSTRACT

Marek´s Disease (MD) is a lymphoproliferative and neuropathic disease of domestic chickens, and less commonly, turkeys and quails, caused by a highly contagious, cell-associated, oncogenic herpesvirus. In Ethiopia MD is believed to be introduced with the exotic and cross-breeds, and has been reported to be a potential threat to both local and exotic breeds in backyard and commercial farming systems. Therefore, the objective of this study was to isolate, identify and molecular characterization of MD virus circulating in the chicken population of the country. Clinical and post-mortem examination was carefully conducted from September, 2013 to December, 2013 in central Ethiopia on clinically diseased chickens suspected of MD virus infection. Representative post-mortem feather follicle and spleen samples were collected under sterile condition and virus isolation was performed using chicken fibroblast cell culture. Cell cultures inoculated with suspension of pathological samples developed characteristic MD virus cytopathic effect of rounding of the cells and small plaques. Further confirmation of the virus was conducted by conventional polymerase chain reaction using MD virus specific primers targeting to amplify the ICP4 gene on twelve samples and revealed the expected band size of 318 base pair of the 11 samples. Eleven positive PCR products were further subjected for sequencing using the amplification primers and the sequence analysis result using the available bioinformatics software's confirmed that the chickens were infected with MD virus. Phylogenetic tree construction was performed to see the genetic relatedness of the present virus isolates with the reference MD virus strains retrieved from the Genbank and revealed that the virus infected the Ethiopian chickens were clustered under Gallid Herpes virus type 2. To our knowledge, the present study is the first report conducted on virus isolation, molecular characterization and sequencing of MD virus isolates in Ethiopia.

Further research work on isolation and molecular characterization of MD virus is recommended by observing chickens at all age groups showing clinical signs of the disease reared under backyard and commercial farming system. Finally, production of an effective MD vaccine in the country which could help in protecting the chickens from the circulating virus type is highly recommended.

Key words: Marek's disease, Marek's disease virus, molecular characterization, polymerse chain reaction, virus isolation, sequencing

1. INTRODUCTION

Ethiopia is one of the few African countries with a significantly large population of chicken, estimated at 38.1 million (CSA, 2009). In villages systems of Ethiopian farmers keep poultry for diverse objectives which include hatching, sale, home consumption, sacrifices (healing ceremonies) and gifts (Yami, 1995; Dessie, 1996). 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). In curbing these problems efforts are being made to enhance poultry productivity and optimize the contribution of chickens to the national economy (Ashenafi, 2000). Accordingly, exotic and cross breeds are being multiplied and distributed to individual farmers to be maintained and produced under improved backyard management system. This is thought to improve the livelihood and nutrition of poor farmers and further to contribute to the national economy at large (Dessie and Ogle, 2001). Attempts are also being made in transforming the production system into commercialized and intensive large-scale system which involves private investment and has brought considerable contribution to the supply of poultry products mainly to the urban areas.

However, it is becoming a growing concern that there is introduction of diseases of various etiologies into several poultry farms concurrent with importation of exotic breeds to backyard chickens. Furthermore, intensification is aggravating the rapid spread of the prevailing infectious diseases between and within poultry farms. The distribution of these exotic breeds to farmers is creating a great treat to the indigenous backyard chickens (Zeleke *et al*., 2005a). Among these threats viral diseases like Newcastle disease (ND), Marek's Disease (MD) and infectious bursal disease (IBD) are the major health constraints inflicting heavy losses (Yami, 1995; Dessie and Ogle, 2001; Zeleke *et al*., 2005a, b).

MD is a highly contagious and economically important oncogenic or paralytic viral disease of poultry and it is becoming a serious problem of the poultry industry of Ethiopia. MD is caused by Marek's disease virus (MDV), an α-herpesvirus that is ubiquitous to poultry, spreads

horizontally, and can cause tumor formation in visceral tissues (Witter and Shat, 2003). MD was first described 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 century, there were increases in the severity of MD, but in general the disease has been controlled successfully by the use of vaccines.

Lobago and Woldemeskel (2004) conducted a study on an outbreak of Marek's disease in a commercial poultry farm in central Ethiopia causing a mortality rate of 46% for the first 14 weeks of the outbreak. Furthermore, according to Duguma *et al.* (2005) the magnitudes of morbidity and mortality on indigenous chickens in Ethiopia were nearly equal, indicating that MD is highly fatal 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 against the threat by MD.

However, despite the growing importance of MD vaccine in Ethiopia, it is being availed by importation from India (INDOVAX Private Limited). In the year 2013/14, NVI imported 1.8 million doses of MD vaccine (HVT FC-126 strain) and distributed for customers (personal communication). These vaccines are produced from MD 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 MD 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 MD virus strains from the local production systems is mandatory. However, previously no study have been conducted in Ethiopia by isolation and sequencing of the MDV. Hence, the findings from this research are therefore steps forward to materialize the production of vaccine from local isolate.

Therefore, the objectives of this study were:

1. To isolate local strains of MD viruses circulating in Ethiopian chickens

2. To undertake molecular characterization and phylogenetic analysis of MD virus isolates.

2. LITERATURE REVIEW

2.1. History of Marek's Disease

MD was first described in 1907 by a veterinarian named József Marek, after whom the disease was named (Marek, 1907). 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. They recounted his detailed description of the clinical signs (paresis of legs and wings) of four roosters and the pathologic changes in one which was killed 5 weeks after signs were first noticed. It was that description of gross enlargement due to mononuclear infiltration of peripheral nerves and spinal nerve roots which encouraged the use of the term Marek's disease (Biggs, 1967).

Several outbreaks of the disease in the United States were reported by Kaupp (1921). According to Biggs (1967), marek's disease was first recorded in Great Britain by Gallowway in 1929.It was also subsequently noted in many other countries.

2.2. Disease definition

Marek’s disease (MD) is a lymphoproliferative and neuropathic disease of domestic chickens, and less commonly, turkeys and quails, caused by a highly contagious, cell-associated, oncogenic herpesvirus (Clanek, 1986; Hennig *et al*.; 2003; Schat and Nair, 2008). The disease is one of the most common diseases affecting poultry flocks worldwide. Although clinical disease is not always apparent in infected flocks, a subclinical disease results decrease in growth rate and egg production may be economically important. Mortality rates can be very high in susceptible birds. MD results in enlarged nerves and in tumor formation in nerve, organ, muscle and epithelial tissue. Clinical signs include paralysis of legs, wings and neck; loss of weight; grey iris or irregular pupil; vision impairment; and the skin around feather follicles can be raised and roughened. Affected birds are more susceptible to other infectious diseases (OIE, 2010). MD commonly appears in 3 to 4 weeks old chickens and gradually

builds to a peak between 12 and 30 weeks of age (Morgan *et al*., 2008). Poultry may be unable to lay eggs or may even die.

2.3. Etiology

In 1967, the agent of Marek's disease was identified as a herpes virus called Marek's disease virus (MDV) (Churchill and Biggs, 1967). Due to its lymphotrpic nature, MDV was originally classified in the family *Herpesviridae* as a member of subfamily *Gammaherpesvirinae* (Chuahan and Roy, 1998). However, on the basis of genomic organization, MDV is currently classified with the viruses of order *Herpesvirales,* family *Herpesviridae*, subfamily *Alphaherpesvirinae*, genus *Mardivirus*, and species *Gallid Herpesvirus* 2 (GaHV-2) (Schat and Nair, 2008; ICTV, 2011).

2.4. Serotypes

MDV strains are classified into 3 serotypes: GaHV-2 (MDV serotype 1: MDV-1), *Gallid herpesvirus* 3 (MDV serotype 2: MDV-2) and *Meleagrid herpesvirus* 1 (MDV serotype 3 or herpesvirus of turkeys (HVT) (Schat and Nair, 2008; Venugopal *et al*., 2001; Walkden-Brown *et al*., 2013). The serotype 1 viruses are further divided by pathotype as mild (m)MDV, virulent (v)MDV, veryvirulent (vv)MDV, and veryvirulent plus (vv+)MDV (Witter and Schat, 2003).

Table 1: Classification of MDV serotypes and their representive strains

MDV serotypes	Pathotypes or strains
Serotype 1 (Pathogenic or oncogenic strains as well as attenuated strain of these viruses)	Very virulent plus(vv+): 648 Very virulent(vv): Md/5,Md/11,Ala-8, R1B Virulent (v): HRPS-16,JM GA Mild (m) virulent: HPRS-B14,Com A Weakly virulent:CU-2,CV1-988
Serotype 2 (Naturally non-pathogenic,non-oncogenic or avirulent strains)	SB-1,HPRS-24,301B/1,HN-1
Serotype 3 (Naturally avirulent strains, non- oncogenic)	HVT (FC126, PB1) (Herps Virus of Tureky)

Source: Shambhu *et al*., 2012

2.5. *Herpesviridae* Family

Herpesviridae is a large family of DNA viruses that cause diseases in animals, including humans (Ryan and Ray, 2004; Sandri-Goldin, 2006). The members of this family are also known as herpesviruses. 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.

2.6. Marek's disease virus

2.6.1. Virus morphology

Herpesviruses all share a common structure-all herpesviruses are composed of relatively large double-stranded, linear DNA genomes encoding 100-200 genes encased 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 and a lipid bilayer membrane called the

envelope. 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.

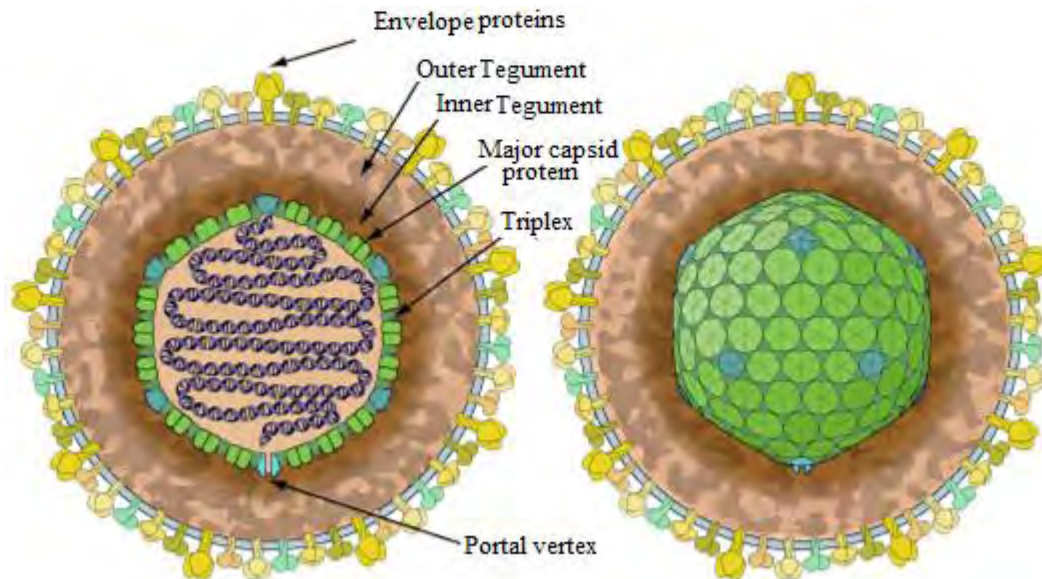

Figure 1. Virion structure of herpesvirus (Source: Viral zone, 2012).

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 (Cebrian *et al*., 1982; Fukuchi *et al*., 1984; Tulman *et al*., 2000). Owing to 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 alphaherpesviruses (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]) (Tulman *et al*., 2000). However, some genes are specific to MDV, such as the gene encoding Meq oncoprotein or pp38 phosphoprotein (Lee *et al*., 2000).

The complete nucleotide sequences of two strains of serotype 1 (oncogenic) MDV (Md5 and GA), one serotype 2 (nononcogenic) strains (HPRS24), and one serotype 3 strain (herpesvirus of turkeys) have been published (Brunovskis and Velicer, 1995; Lee *et al*., 2000; Tulman *et al*., 2000; Afonso *et al*., 2001).

The ICP4 gene is encoded in the inverted repeat flanking the unique short region (Anderson et al.,1992). The coding sequence, which is 4,245 nucleotides, is highly conserved among various strains (Anderson et al.,1992, Spatz et al.,2007). There are many transcriptional regulatory sites upstream and downstream of the ICP4 translational start site (Anderson et al.,1992). A long open reading frame is positioned 5' to and in frame with the conventional ICP4 coding sequence (Anderson et al.,1992). 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 (Pratt et al.,1994).

Tulman *et al*. (2000) presented the first complete genomic sequence, with analysis of a very virulent strain of MDV serotype-1 Md5. The genome 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 colinear 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.

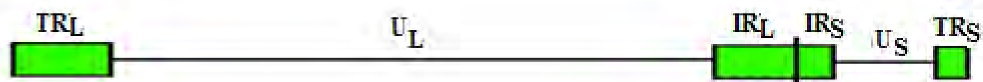

Figure 2. Genome organization of Marek's disease herpesvirus. Unique long(U_L) and Short (U_S) regions are flanked by inverted and terminal repeat long and short regions (IR_L, IR_S, TR_L, and TR_S). (Source: Tulman *et al*., 2000).

2.7. Pathogenesis

The pathogenesis of marek's disease can be sequentially divided into 3 phases: early cytolytic phase, latent phase, and secondary cytolytic phase with immunosuppression and tumor development. In the early cytolytic phase, MDV-1 causes lytic infection of lymphoid cells, mainly B cells that last for up to six days after infection (Shek *et al.,* 1983). Then, this cytolytic infection induces the activation of T cells, and MDV establishes latency in a part of the activated $CD4^+$ T cells at 1–2 weeks after infection. In the latent phase, infected chickens show no clinical signs, but cellular immunity is continually inhibited by apoptosis of $CD4^+$ T cells, CD8-down regulation in $CD8^+$ T cells, decrease in the responsiveness to the stimulation through T cell receptor (TCR) in $CD4^+$ and $CD8^+$ T cells and MHC class I-down regulation at 2–3 weeks after infection (Morimura *et al.,* 1996; Hunt *et al.,* 2001). In the secondary cytolytic phase, MDV-1 transforms a few latently infected $CD4^+$ T cells, and develops malignant lymphomas. The main targets for the transformation by MDV-1 are $CD4^+$ T cells, suggesting that latent infection in this T cell subset is intimately related to the subsequent transformation by MDV-1 (Schat and Nair, 2008).

The natural route of MDV infection is via respiratory tract following inhalation of infectious cell-free MDV present in dander shed from feather follicles. All chickens are susceptible to infection with MDV. The role of the lung in MD pathogenesis is not clear and it is presumed that phagocytes present in the lung pick up the virus and carry it to lymphoid organs such as bursa of Fabricius, thymus and spleen (Phillips *et al*., 1972). MDV reaches the lymphoid organs within 2–3 days after infection. In the lymphoid organs, infection of lymphocytes

seems to be assisted by splenic ellipsoid-associated reticulum cells (Jeurissen *et al.*, 1992). A productive, cytolytic, infection is shortly established in the lymphoid organs, and is particularly evident between 5 and 6 days post-infection (dpi). The initial target cells for the cytolytic infection are B lymphocytes (Shek *et al*., 1982). The necrosis of B cells in the lymphoid organs elicits an immune response and the subsequent recruitment of numerous inflammatory cells, especially macrophages, T and B lymphocytes and some heterophils. The activation of T cells is of particular relevance in the pathogenesis of MDV since resting T cells are refractory to MDV infection. Activated T cells can also support productive infection but in most cases, infection becomes latent after T cells are infected (Calnek *et al*., 1984). Until recently, macrophages were considered to be resistant to MDV infection and replication, but provided support for the role of macrophages by demonstrating the presence of MDV transcripts in the cytoplasm and nucleus of splenic macrophages after infection with a hyper virulent strain of MDV (Barrow *et al.*, 2001; Barrow *et al.*, 2003).

The switch from cytolytic infection to latency occurs very fast and by 7–8 days, there is minimal evidence of cytolytic infection in the lymphoid organs. However, more virulent viruses seem to induce longer cytolytic infection in the lymphoid organs (Calnek *et al*., 1998; Barrow and Venugopal, 1999). Latent infection can be detected by 7–8 days post-infection not only in lymphoid organs but also in peripheral blood lymphocytes. Latently infected peripheral blood lymphocytes probably are the disseminators of MDV to other tissues of the chickens. As early as 6 days post-infection, MDV can be detected in the brain, peripheral nerves and eye (Gimeno *et al.*, 2001; Gimeno and Silva, 2006). Depending on the virulence of the virus and the genetic resistance of the chickens, MDV can reactivate in some of the lymphocytes infiltrating the brain tissue and induce an acute inflammatory response characterized by vasculitis and vasogenic edema that are responsible for the clinical signs of transient paralysis. Highly virulent MDVs (vv and vv+) induce a more severe vasculitis that leads to high mortality (Gimeno *et al.*, 2002). Extensive damage to the brain is concomitant with, and probably caused by, an increase in nitric-oxide levels in both plasma and brain tissue (Jarosinski *et al.*, 2002; Jarosinski *et al.*, 2005; Abdul-Careem *et al.*, 2006).

A second wave of cytolytic infection is detected in most tissues of epithelial origin by the end of the second week after infection. Because of its relevance in transmission, productive infection within the feather follicle epithelium (FFE) is particularly important. Replication of MDV in the FFE occurs in genetically resistant as well as susceptible birds regardless of MDV virulence. Most likely, MDV is transferred to the FFE by infected lymphocytes within dermal perifollicular lymphoid aggregates that develop as early as 7 days post-infection. Transmission of MDV from the FFE will continue throughout the life of the chicken. Latently infected T cells can become transformed leading to the development of lymphomas. Transformation can occur in lymphocytes infiltrating any tissue. Therefore, MD produces significant clinical disease as the result of tumors lesions in many visceral tissues with predilection in nerves and skin, and the other clinical manifestations can be numerous and varied, including paralysis, skin lesions, atrophy of the thymus and bursa of Fabricius, immunosuppression and high mortality (Kreager, 1998; Kingham *et al*., 2001; Burgess *et al*., 2004). Xing and Schat (2000) investigated the effects of MDV infection on transcription of a number of cytokines, both in vitro and in vivo. IFN-γ transcription is increased from as early as 3 dpi until at least 15 dpi. There is also upregulation of interleukin-1β (IL-1β), IFN-α, and, after 6 dpi, inducible nitric oxide synthase (iNOS). Xing and Schat (2000) proposed that IFN-γ plays a pivotal role in the early pathogenesis and immune responses to MDV infection.

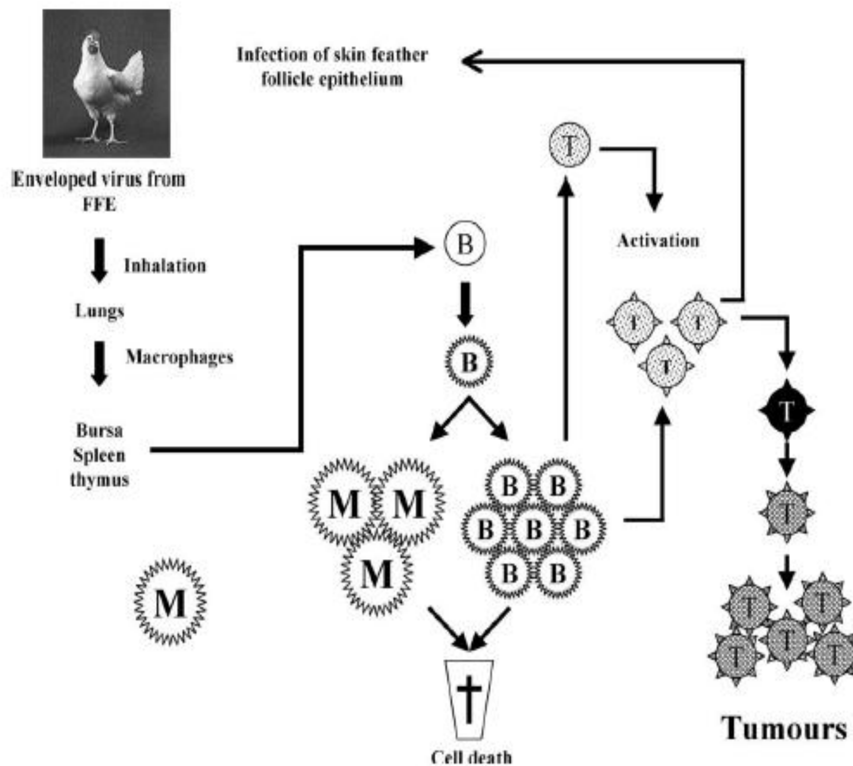

Figure 3: Pathogenesis of MD. Birds get infected by the inhalation of infectious virus shed from the feather follicle epithelium (FFE). After initial replication in the lungs, the virus replicates in the lymphoid organs. B-cells (B) and macrophages (M) undergo a lytic infection, resulting in the activation 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: Venugopal, 2005)

2.7.1. 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 (Calnek *et al.,* 1970). The mechanisms of entry of cell-free virus into cells have been little studied and the virus receptors have not been identified. 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 (Vengopal and payne, 1995).

The MDV genome codes for 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 jun/-fos family of transcriptional factors (Jones *et al*., 1992). Meq is consistently expressed in all MDV transformed cells, suggesting that it may play an important role in transformation (Jones *et al*.,1992). pp38 is a phosphoprotein expressed in both lytically infected and tumor cells (Cui *et al*.,1991). The function of this protein is still not clear but has been suggested to be involved in the maintenance of transformation (Xie *et al*.,1996). Understanding the role these proteins play in oncogenesis requires the introduction of mutations in the viral genome.

2.7.2. 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 (Graham, 1976; Kreager, 1998). 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 (Josipovic,1990; Izumiya *et al*., 2001). Very soon after infection of the respiratory tract, cell associated viremia can be detected in the blood, reaching a peak about eight days later (Silva *et al*., 2004). Macrophages carry and distribute MDV all over the body infecting sensitive cells and causing lymphocyte transformations (Josipovic, 1990). Once the virus is shed into the environment, it can remain infectious for many months (Kreager, 1998; Rodriguez *et al*., 2007).

2.8. Diagnosis of Marek's Disease

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 chicken, thus one must consider both the diagnosis of the disease/tumors (pathological diagnosis) and of the virus (etiological diagnosis). 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 (Witter *et al*., 2010).

The presence of nodules on the internal organs may also suggest Marek's disease but further testing is required for confirmation. This is done through histological demonstration of lymphomatous infiltration into the affected tissue. A range of leukocytes can be involved, including lymphocytic cell lines such as large lymphocyte, lymphoblast, primitive reticular cells and occasional plasma cells as well as macrophage and plasma cells. The T-cells are involved in the malignancy, showing neoplastic changes with evidence of mitosis. The lymphomatous infiltrates need to be differentiated from other conditions that affect poultry including Lymphoid Leukosis and Reticuloendotheliosis. 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 (OIE, 2010).

2.8.1. Conventional Marek's Disease diagnosis

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. Other observations include grey eye (iris) or irregular pupil, vision impairment,blindness, skin lesions and immunosuppression. Laboratrory conformition is done by virus isolation in susceptible (newly hatched) chicks, embryonated eggs and tissue cultures and subsequent identification (Kataria *et al*., 2005). MDV can be isolated in chicken embryos by yolk sac route (4-5 days embryo) and later examining their chorioallantoic membranes (CAM) on 18th day of incubation for the 'pock lesions' (whitish raised nodules). Infected embryos also show atrophy of muscles and curling.MD virus can also be isolated in chicken kideny cells culture and chicken 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.Viral antigen can be detected in feather tips, follicle epithelium and infected lymphoid tissue by Agar Gel Precipitation Test (AGTP), Fluorescent Antibody Technique (FAT), Immunoperoxide Test (IPT) and Enzyme-linked Immunosorbent Assay (ELISA) (Shat and Nair, 2008). Immunohistochemistry can be effectively used to demonstrate MDV proteins especially in all MD tumours.

2.8.2. Recent advances in Marek's disease diagnosis

2.8.2.1. Polymerase chain reaction

The full length genomic sequences of MDV 1 (GenBank accesion numbers: strain Md5; AF243438, GA; AF147806,Mdl 1; AY510475,CV1988; DQ530348), MDV2(GenBank accession number: strain SB-1; HQ840738,HPRS24; AB04935 (Izumiya *et al*., 2001) and MDV 3 (GenBank accession number: strain FC126; AF291866) are available now. This enables the PCR-based diagnostic methods for serotype specific detection of MDV. 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; Handberg *et al*., 2001).

2.8.2.2. Nested polymerase chain reaction

Specific detection of meq oncogene of MDV 1 in infected spleen cells, feather tips and peripheral blood mononuclear cells by nested PCR have been developed (Lee *et al*., 2000; Murata *et al*., 2007).

2.8.2.3. Mulltiplex polymerase chain reaction

Simultaneous detection of MDV 1, avian leukosis virus and reticculoendotheliosis virus in tumour tissues of naturally infected chickens and turkeys has been developed using multiplex PCR (Gopal *et al*., 2012).

2.8.2.4. 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 (Baigent *et al*., 2005; Abdul-Careem *et al*., 2006; Islam *et al*., 2006). 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.

2.8.2.5. Loop mediated isothermal amplification technique

Loop mediated isothermal amplification technique techinque for rapid detection of marek's disease virus meq gene in feathers of affected birds has been developed lately (Wozniakowski *et al*., 2011; Angamuthu *et al*., 2012; Wei *et al*., 2012). 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 (Wei *et al*., 2012). The Loop mediated isothermal amplification technique utilizes three different sets of primers binding to six different sequences thus adding more specificty, the reaction is

carried out at isothermal conditions and the products can be visualized by the naked eye (Goto *et al*., 2009; Notomi *et al*., 2000).

2.9. Distribution

Marek's disease virus has a worldwide distribution,occurring in all poultry producing areas.Marek's disease is responsible for great economic loses to the poultry industry world wide. Sporadic outbreaks of MD have been reported recently throughout the world even in vaccinated flocks (Powell and Lombardini, 1986; Kuria *et al*., 2001, Okwor and Eze, 2011), including India (Rajkhowa, 2005; Bineesh *et al*., 2007; Jadhaw *et al*., 2007; Kamaldeep *et al*., 2007; Raja *et al*., 2009; Arulmozhi *et al*., 2011; Gopal *et al*., 2012).

Dr. Jozef Marek first recognized the disease as a paralysis of roosters in the 1907. MD almost devastated the poultry industry in the 1960s but the disease was brought under control after Marek's disease Herps Virus of Turkey (HVT) was identical and live vaccines were developed in 1970's. Thereafter, variant MD viruses evolved with increased pathogenicity.Subsequently, many MD outbreaks have been reported worldwide and new vaccines developed to combat MD viruses with higher virulence. Earlier it is considered as paralytic disease but now-a-days, it is manifested as an acute disease with tumours in multiple visceral organs. Today there are evolving highly pathogenic isolates of MDV around the world capable of overwhelming the protection from currently employed vaccines. Thus, MD poses a big challenge to the welfare and wellbeing of the poultry with increased condemnation of carcass, loss of productivity and quality products, leading to huge economic losses.It is also an immunosuppressive disease and causes increased susceptibility to other infections (Shambhu *et al*., 2012).

Gimeno (2004) used a questionnaire to analyze the incidence of MD in 55 countries. Only five countries, including Peru in Latin America, reported economic losses caused by MD since the 1990´s. In the America´s 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. In addition to the America´s, outbreaks were reported in a few European countries including Russia with economic losses

and China. MD is only occasionally diagnosed in the Americas and the incidence is decreasing in most of South, Central and North America. Exceptions are Bolivia, Columbia, Peru, Uruguay and Venezuela. 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. 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. Usually MD includes neural lesions, visceral and skin tumors and perhaps transient paralysis in commercial flocks, but until recently there were no reports of early mortality syndrome (EMS) outside of experimental infections.

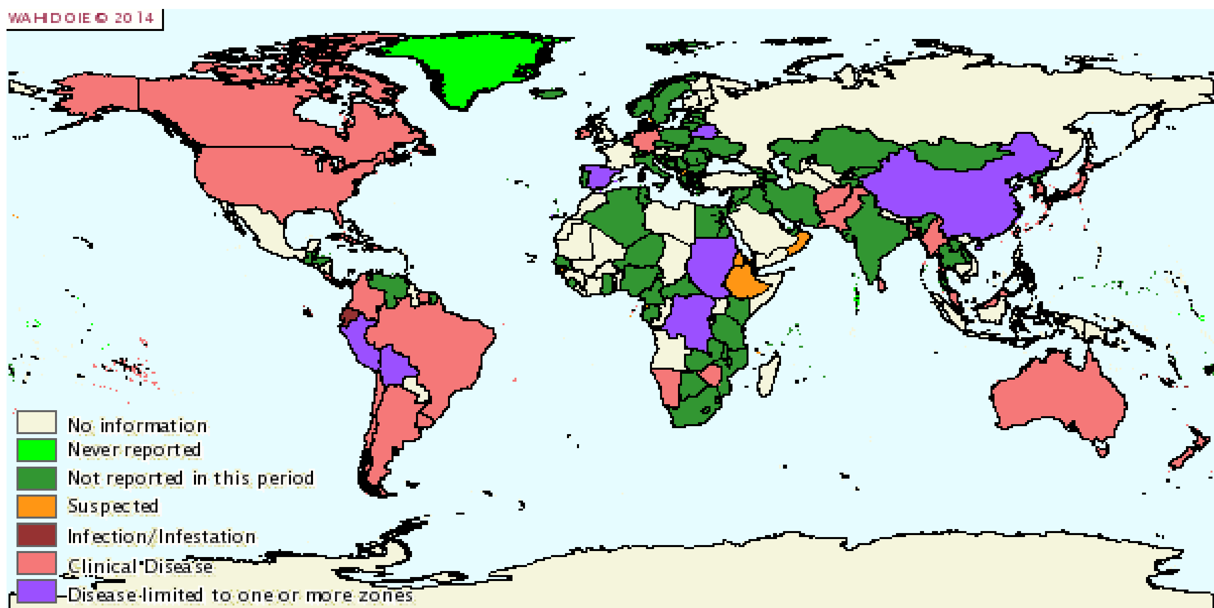

Figure 4. Marek's disease distribution map (Source: OIE-WAHID, 2011).

2.9.1. Status of marek's disease in Ethiopia

Marek's disease is becoming a serious problem of the poultry industry of Ethiopia Lobago and Woldemeskel (2004) conducted a study on an outbreak of Marek's disease in a commercial

poultry farm containing 8500 chicken 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. According to Duguma *et al.* (2005) the magnitudes of morbidity and mortality on indigenous chickens in Ethiopia were nearly equal, indicating that MD is highly fatal to the local breeds. The study was conducted at Deber zeit Agricultural Research Center, Central Ethiopia ,on local chickens were purchased from different geographical areas of the country. In all cases the situation of the disease at small scale commercial flocks, and back yard poultry farms indicate the disease is widely distributed in the county and call for detail prevalence and epidemiological investigation.

2.10. Control and Prevention of Marek's Disease

2.10.1. Vaccination

In 1967, the first isolation of MDV was successfully achieved (Churchill and Biggs, 1967; Nazerian *et al.*, 1968), and enormous efforts for vaccine-development were independently performed. In early 1970s, neonatal vaccination with live apathogenic MDV was found to prevent lymphoma formation, and since then, MD was effectively controlled by vaccination. Currently, all three serotypes of MDV, serotype 1 (attenuated strains) (Rispens *et al.,* 1972), serotype 2 (non-pathogenic strain) (Zander *et al.*, 1972; Schat and Calnek,1978) and serotype 3 (herpes virus of turkey, HVT) (Okazaki *et al.*, 1970; Witter *et al*,1970; Purchase *et al.*, 1971) are used as vaccines.

Live virus vaccines, used since 1970, remain the basis of disease control programmes. 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. 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 (Rispens), and R2/23 (Md 11/75) and serotype 3,2,2,1,1,1,1 respectively) (Payne and Venugopal, 2000).

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 CV1988 strain Rispens vaccines and modified versions are widely used in many countries and appear to be effective against some of the vv+MDV pathotypes. Although MD vaccines have been successful in controlling major losses from the disease, threat of vaccine failure has continued to cause concern. According to Payne and Venugopal (2001) 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 (Witter, 1997). The development of more effective vaccines through recombinant DNA De Laney technology (Ross, 1998), and the use of immunomodulatory approaches to enhance the response to vaccines should improve vaccination strategies in the future.

2.10.2. 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. 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 (Murphy *et al*., 1999, Venugopal *et al.,* 2001).

2.10.3. 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. 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 (Payne and Venugopal, 2000).

3. MATERIALS AND METHODS

3.1. Study Area

Accordingly, clinical materials (spleens and feather follicles) were collected from MD suspected chicken reared under semi-intensive and intensive poultry farms located at Debre zeit, Mojo, Addis Ababa, and Sebeta (Figure 5). Debre zeit (8° 44' N, 39° 02' E) is located in mid-highland having 1900 meter above sea level (m.a.s.l.) and is characterized by moderate rainfall (851mm mean annual rainfall), 17ºC average mean temperature (Bemnet *et al.,* 2003). While Addis Ababa is known to have daily average temperature ranging from 9.9 to 24.6ºC with mean annual rain fall of 1224 mm and elevated 2110 m.a.s.l. (Tesfaye, 2009). Mojo district is located at 70km from Addis Ababa in East Shewa zone of Oromia Region, Ethiopia. Most the district of Mojo altitude ranging from 1500 to 2300 m.a.s.l and its mean monthly temperature of the area ranges from 22ºC to 34ºC. As mentioned in Dawit (2012), Sebeta is located 25Km South West of Addis Ababa and situated at 8° 55' N and 38° 37' E. it has an elevation of 2356 meter above sea level and has annual rainfall of about 1650mm. Its mean annual maximum and minimum temperature ranges between 8ºC to 19ºC respectively.

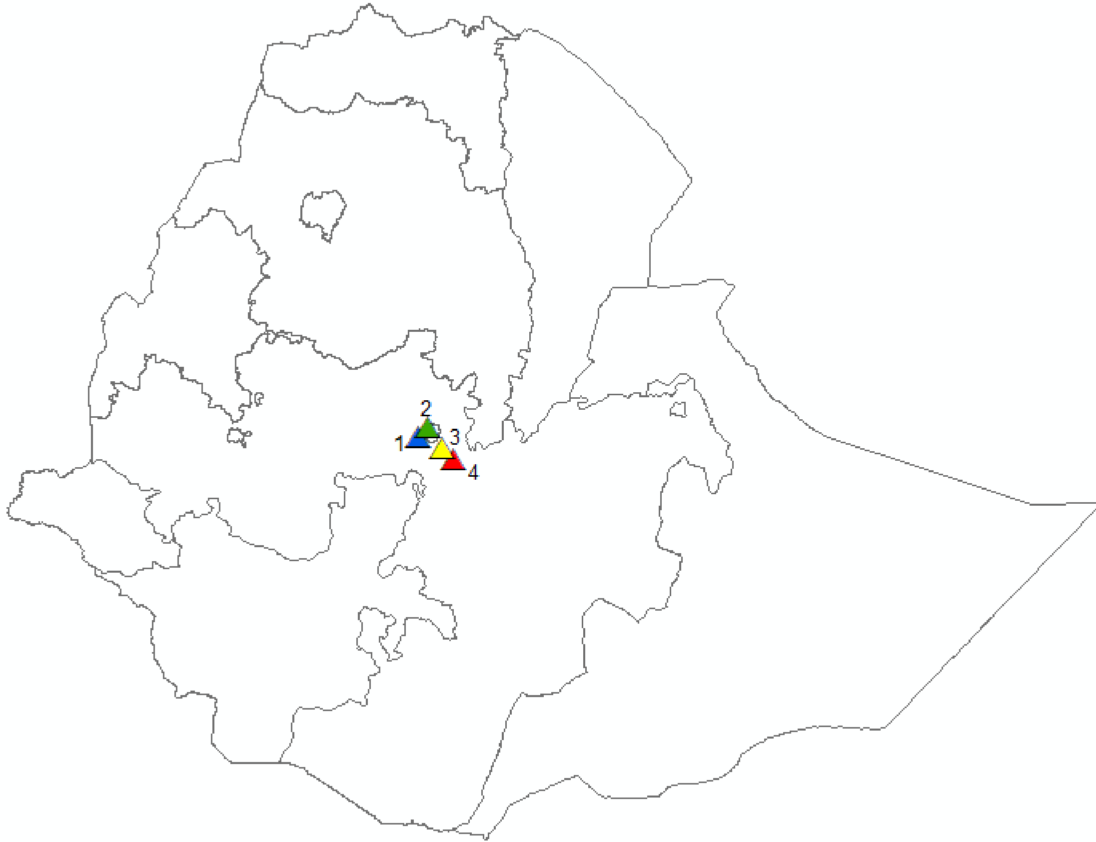

Figure 5. Map of Ethiopia showing the study areas where MD outbreak samples were collected from clinically diseased chickens. Where 1 = Sebeta, 2 = Addis Ababa, 3 = Debre zeit, and 4 = Mojo.

3.2. Study Animals

The study was conducted in chickens that had experienced outbreaks of marek's disease. Chickens of all ages and breeds reared under semi-intensive and intensive production and management system were included in the study.

3.3. Study Design

The study was focused on an outbreak investigation of marek's disease. The result of this study was based on molecular and phylogentic characterization of marek's disease virus.

3.4. Sample Collection and Transportation

Sample collection were undertaken in two ways, i.e., from clinically sick chicken suspected of marek's disease and brought to the NVI Research and Diagnostic laboratory by the poultry owners or attendant for disease diagnosis or from field based on outbreak reports. Suspected clinical samples were collected following careful examination of individual cases. For virus isolation, spleen and feather follicle samples were collected aseptically from MD suspected clinically sick chickens after killing in the post mortem facilities of the institute. Samples were placed in sterile and labelled universal bottles and transported using cold-chain to the diagnostic laboratory of the NVI. In the laboratory samples were either processed immediately or kept at -80ºC awaiting processing (Witter, 1997; OIE, 2010).

3.5. Laboratory Investigation

3.5.1. Cell culture

Primary chicken embryo fibroblast (CEF) cells were prepared inside the Biosafety cabinet class II from 11 day old Specific Pathogen Free (SPF) embryos (Delany *et al*.,1998; Schat and Purchase, 1998; Tan *et al*.,2008). Accordingly, embryonated SPF eggs were disinfected with 70% ethanol and shell opened to remove embryo. After removing the head and legs, the body was washed three times with sterile PBS. The body was then fragmented by carefully chopping and washed three times using sterile PBS to remove red blood cells. Pre-warmed, at 37ºC, 0.25% trypsin solution was added on the chopped material and placed into an incubator (at 37ºC) and let for 15 minutes with slow agitation. The suspension was centrifuged for 10 minutes at 1000 rpm. The supernatant was poured off and the cells were resuspended with Glasgow minimum essential medium (GMEM). The cell suspension was transferred into a 25cm^2 plastic tissue culture flask and kept at 37ºC incubator.

3.5.2. Virus isolation

3.5.2.1 Virus isolation from spleen

Spleen samples were first chopped into small pieces using sterile scissor inside the Biosafety cabinet class II and further ground in to smaller pieces using sterile mortar and pestle in the virology laboratory of the institute. Three to five spleen samples collected from the same outbreak farm were pooled and processed to increase the chance of virus isolation. A 10% (w/v) suspension of spleen samples were prepared in sterile phosphate buffer saline (PBS) supplemented with penicillin (100 IU/ml) and streptomycin (1000 μg/ml). The suspension was transferred into sterile tube and centrifuged at 2000 rpm,+4°C 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 37ºC. Cultures were observed daily using inverted microscope for up to 1 week to check for the presence of cytopathic effect (CPE) characteristic for MDV (Ross *et al*.,1972). Those that had not showed CPE were blindly passaged up to the third passage. Samples did not develop CPE until the third blind passage was considered as negative, where as samples revealed characteristics CPE were considered as positive and kept at -20°C for further molecular analysis.

3.5.2.2. Virus isolation from feather

Five feather follicles from individual affected chicken were collected and three to five chicken feather from the same outbreak farm were pooled and processed inside the Biosafety cabinet class II as one sample to increase the chance of virus isolation. A 1:10 (wt/vol) suspension of feather tips in SPGA/EDTA (sucrose, phosphate, glutamate and albumin/ethylenediamine tetra-acetic acid) buffer was homogenized for 3-5 minutes and then sonicated for 2 minutes. A suspension was supplemented with penicillin (100 IU/ml) and streptomycin (1000 μg/ml). The suspension was transferred into sterile tube and centrifuged at 2000 rpm,4ºC for 10 minutes. Supernatants were harvested and inoculated onto confluent primary chicken fibroblast cells in maintenance GMEM supplemented with 2% bovine foetal calf serum and incubated at 37ºC. Cultures were observed daily using inverted microscope for up to 1 week to

check for the presence of characteristic CPE for MDV. Those that had not showed CPE were blindly passaged up to the third passage. Samples which did not develop any CPE until the third blind passage was considered as negative, where as samples revealed characteristics CPE were considered as positive and kept at -20ºC for further analysis by molecular and sequencing techniques (Wozniakowski *et al.*,2011).

3.6. DNA Extraction

DNA extraction was conducted in the molecular biology laboratory of the National Veterinary Institute. Extraction of DNA from 10% (w/v) tissue sample and/or cell culture homogenate was carried out using DNeasy Blood and Tissue Kit (Qiagen, Germany) following the manufacturer's instruction. Accordingly, 200µl tissue suspension was transferred into a labelled 1.5 ml microcentrifuge tube. 20 µl proteinase K and 200 µl Buffer AL was added for each tube and mixed by vortexing and incubated at 56ºC for 30 minutes (until completely lysed). 200µl 96% ethanol was added per tube and mixed thoroughly gently by vortexing. The mixture was transferred to a labelled DNeasy mini spin column placed in a 2ml collection tube and centrifuged for 1 minute at 12000rpm. The collection tube was changed by new one and 500µl Buffer AW1 was added into the spin column and centrifuged for 1 minute at 12000rpm. The collection tube was again changed by new tube and 500µl Buffer AW2 was added and centrifuged for 3minutes at 20000rpm. Finally, the spin column was transferred into a labelled 1.5ml eppendorf tube and 40 µl Buffer AE was added to the column and the nucleic acids bound to the silica membrane was eluted and the eluted DNA yield was used for PCR amplification.

3.7. Polymerase Chain Reaction

Polymerase chain reaction (PCR) offers faster and more reliable recommended test as diagnostic tool of Marek's disease (Handberg *et al.*, 2001; Islam *et al.*, 2004; Baigent *et al.*, 2005; Islam *et al.*, 2006; Renz *et al.*, 2006). PCR was performed targeting the amplification of the partial sequence of ICP4 gene of MD virus (318bp) using the forward primer M1.1(5'-GGATCGCCCACCACGATTACTACC-3') and reverse primer M1.8 (5'-

ACTGCCTCACACAACCTCATCTCC-3') as described by Kalyani et al., 2010. The primers were synthesized by VBC Biotech (Vienna, Austria) and purified by reverse phase high-performance liquid chromatography. The PCR was carried out in a final reaction volume of 20 µL using a 200 µL capacity thin wall PCR tube containing 10× PCR buffer, 25 mM $MgCl_2$, 200 µM of the four dNTPs, 5 pmol/µl of each primer, 1U Taq DNA polymerase, and 4 µL template DNA. The PCR tubes were transferred to a thermal cycler (2720, Applied BioSystems). The PCR protocol was performed with an initial denaturation at 95°C for 5min, followed by 35 cycles of denaturation at 95°C, annealing at 58°C and extension at 72°C each for 30sec, and final extension at 72°C for 5min.

3.8. Agarose Gel Electrophoresis of Polymerase Chain Reaction Products

The polymerase chain reaction products were analysed with 1.5% agarose gel stained with gel red. Briefly, 5µl each PCR products was mixed with 6X loading buffer and loaded into separate well of the prepared in pre-prepared gel and 100bp DNA molecular marker was also added onto the first lane and run at 100 volt for about 60 minutes in electrophoresis apparatus. The DNA band was visualized by gel documentation and the size of the PCR products was estimated by comparing with the band size of the molecular marker.

3.9. Sequencing and Phylogenetic Analysis

Polymerase chain reaction positive product was purified individually using Wizard® SV Gel and PCR product purification kit (Promega, Germany) following the manufacturer's instruction. The purified virus DNA concentration was quantified using microvolume spectrophotometer (Nanodrop 2000c, USA). The concentration of the quantified purified PCR product was adjusted following the requirements set by the sequencing company. Either forward or reverse sequencing primers were added into the labelled eppendorf tube containing the quantified DNA. The eppendorf tubes containing mixture of DNA and primer were sent to the sequencing service company (LGC Genomics, Germany). The raw sequence data was edited, blasted, aligned and phylogenetic tree was constructed including the reference strain

sequences (n=9) retrieved from the Genbank (Table 2) using the available Bioinformatics tools (BioEdit, Vector NTI 10, MEGA5.1, NCBI and others).

Table 2. Reference MD virus sequences retrieved from the Genbank

Number	GenBank accession number
1	AF147806
2	AF193006
3	DQ530348
4	EF523390
5	EU499381
6	JF742597
7	JN034588
8	JQ314003
9	JX844666

3.10. Data Analysis

All generated marek's disease virus ICP4 partial gene nucleotide raw sequence data were cleaned and contig was formed using VectorNTI software (Invitrogen). Sequences were edited using the applications of BioEdit software (Hall,1999). Marek's disease virus reference sequences were retrieved from the NCBI databank. Multiple sequence alignment was conducted including the present field outbreak isolates together with MD virus reference sequences retrieved from the databank using ClustalW application (Thompson *et al*., 1994). A homologous region of 318bp nucleotide length corresponding to the partial sequence of ICP4 gene was used for phylogenetic tree construction using the different applications found in MEGA5.1 software (Tamura *et al.,* 2011).

4. RESULTS

4.1. Field clinical examination

A total of 75 chickens: 57 from Debre zeit, 10 from Mojo, 1 from Addis Ababa and 7 from Sebetta showing clinical signs of marek's disease (Figure 5) were examined and representative spleen and feather samples were collected (Table 3).

Table 3. Clinically diseased chickens examined for MD

Town	Date of collection	Type of organ		Farming system
		Spleen	Feather	
Deberzeit	23/09/2013	12	60	Semi-intensive
Deberzeit	07/11/2013	10	50	Intensive
Deberzeit	08/11/2013	13	65	Semi -intensive
Deberzeit	14/11/2013	10	50	Semi-intensive
Deberzeit	23/12/2013	12	60	Semi-intensive
Deberzeit	23/12/2013	-	10	Semi-intensive
Mojo	22/11/2013	3	15	Semi-intensive
Mojo	10/12/2013	3	15	Semi-intensive
Mojo	27/12/2013	4	20	Semi-intensive
Addis Ababa	22/11/2013	1	5	Semi-intensive
Sebeta	10/12/2013	3	15	Semi-intensive
Sebeta	27/11/2013	4	20	Semi-intensive
Total		**75**	**375**	

Clinical examination were conducted on MD suspected chickens from Sebeta, Addis Ababa, Debre zeit, and Mojo. The diseased birds showed clinical signs of paralysis (leg and wing), weight loss, labored breathing and depression. Enlargement of the spleen was seen in most examined cases (Figure 6).

Figure 6. Clinically diseased chickens suspected of MDV infection

4.2. Marek's Disease Virus Isolation

A total of 450 samples, 75 spleens and 375 feather follicles, were collected from 75 chickens of different outbreaks. Samples were collected from different poultry farms (57 spleen and 285 feather) located in Debre zeit, from Mojo (10 spleen and 50 feather), from Addis Ababa (1 spleen and 5 feather) as well as from Sebetta (7 spleen and 35 feather). Out of 12 pooled spleen samples, viruses were isolated from 11 (91.6%) preparations and also out of 16 pooled feather samples, viruses were isolated from 14 (87.5%). All the isolates were grown and developed CPEs with visible small plaques on CEF cell cultures starting from the 4th days of 3rd blind passage. An early CPE was seen as small round cells reflecting light waves. These cells formed foci and syncytia later that detached from the wall of cell culture flask causing formation of plaque (Figure 7).

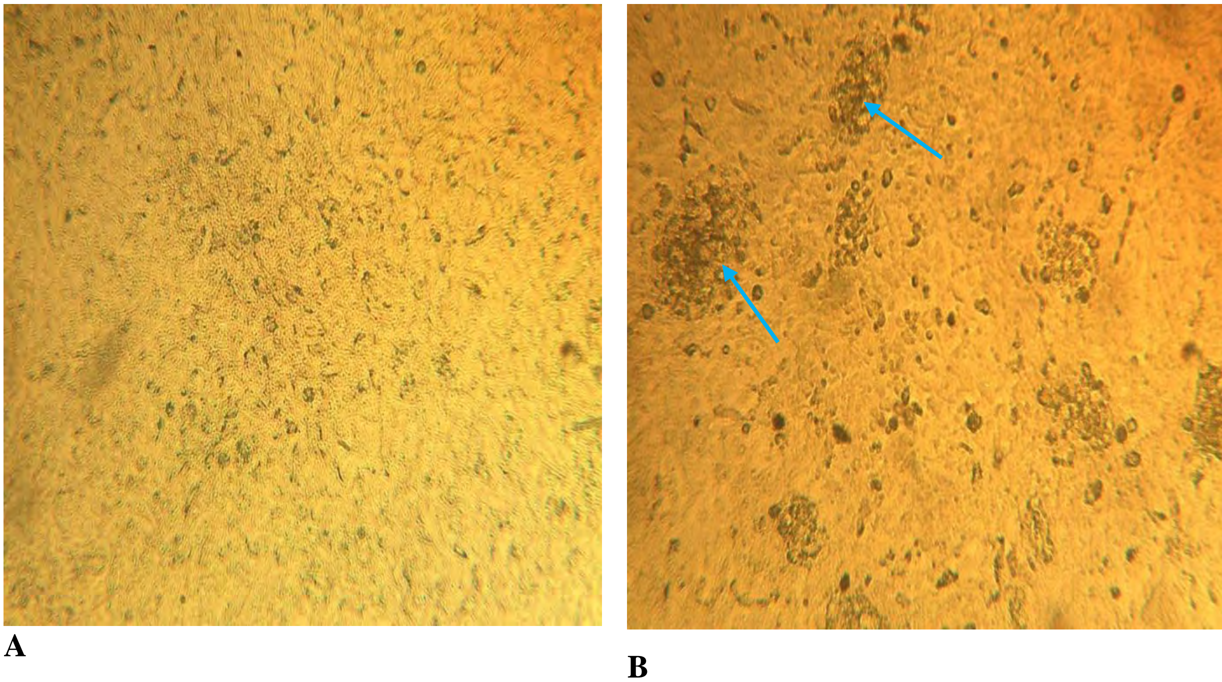

Figure 7. MD virus growth on chicken embryo fibroblast cells. where (A) Confluent monolayer of chicken embryo fibroblast (CEF) cell grown in GMEM growth medium after 48 hours of culture; (B) Characteristic cytopathic effect (CPE) of MD virus (blue arrow) on CEF cell developed after 4th days post-infection of third blind passage.

4.3. Detection of viral DNA by conventional Polymerase Chain Reaction

Polymerase chain reaction was performed targeting to amplify a 318bp fragment of ICP4 gene of MDV using the forward primer M1.1 and reverse primer M1.8. This step allowed us to reach a definitive diagnosis of the causative agent of the outbreak. Eleven pooled spleen as well as feather samples suspension was tested for MDV genome. All samples showed the presence of 318bp PCR products (Figure 8). The samples which were negative for virus isolation were also found negative in PCR (Table 4).

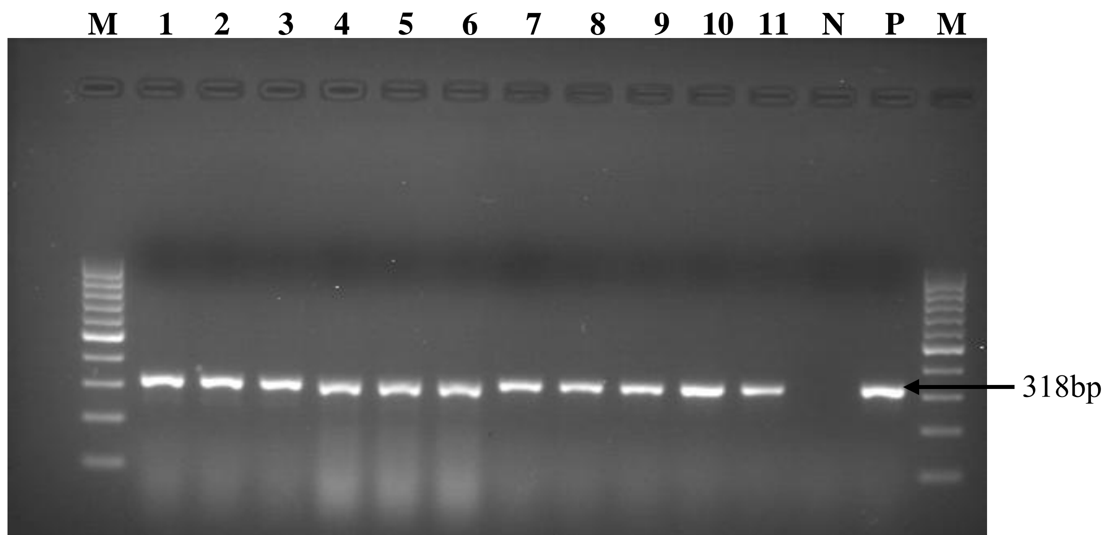

Figure 8. Agarose gel electrophoresis pattern of MDV partial ICP4 gene PCR products (approximately 318bp). Where M=100bp DNA molecular markers (Fermentas); 1- 11: Field samples; N: Negative control; P: positive control.

Table 4. Outbreak samples which revealed positive result for MDV tested by gene specific PCR.

Town	**No. of pooled samples tested**	**Result**	
		Positive	**Negative**
Debre zeit	6	5	1
Mojo	3	3	-
Addis Ababa	1	1	-
Sebeta	2	2	-
Total	**12**	**11**	**1**

4.4. Sequencing and Phylogenetic Tree Analysis

All eleven MDV Ethiopian isolates examined in this study fell into a serotype 1, Gallid Herpesvirus-2 (Fig.9). The identified Ethiopian isolates MDV-Sebeta-01-2013-ICP4, MDV-Mojo-03-2013-ICP4, MDV-Mojo-01-2013-ICP4, MDV-Addis Ababa-01-2013-ICP4, MDV-Deber Zeit-03-2013-ICP4, MDV-Deber Zeit-02-2013-ICP4 and MDV-Deber Zeit-01-2013-ICP4 indicates that they all most closely related to JN034588 Gallid-herpesvirus -2, AF147806 Gallid-herpesvirus -2, and JX844666 Gallid-herpesvirus -2. Whereas MDV-Deber Zeit-05-2013-ICP4, MDV-Sebeta-02-2013-ICP4 and MDV-Deber Zeit -04-2013-ICP4 were showed less variation with above mentioned Ethiopian isolates which supported by a bootstrap value 65% (Fig.9).

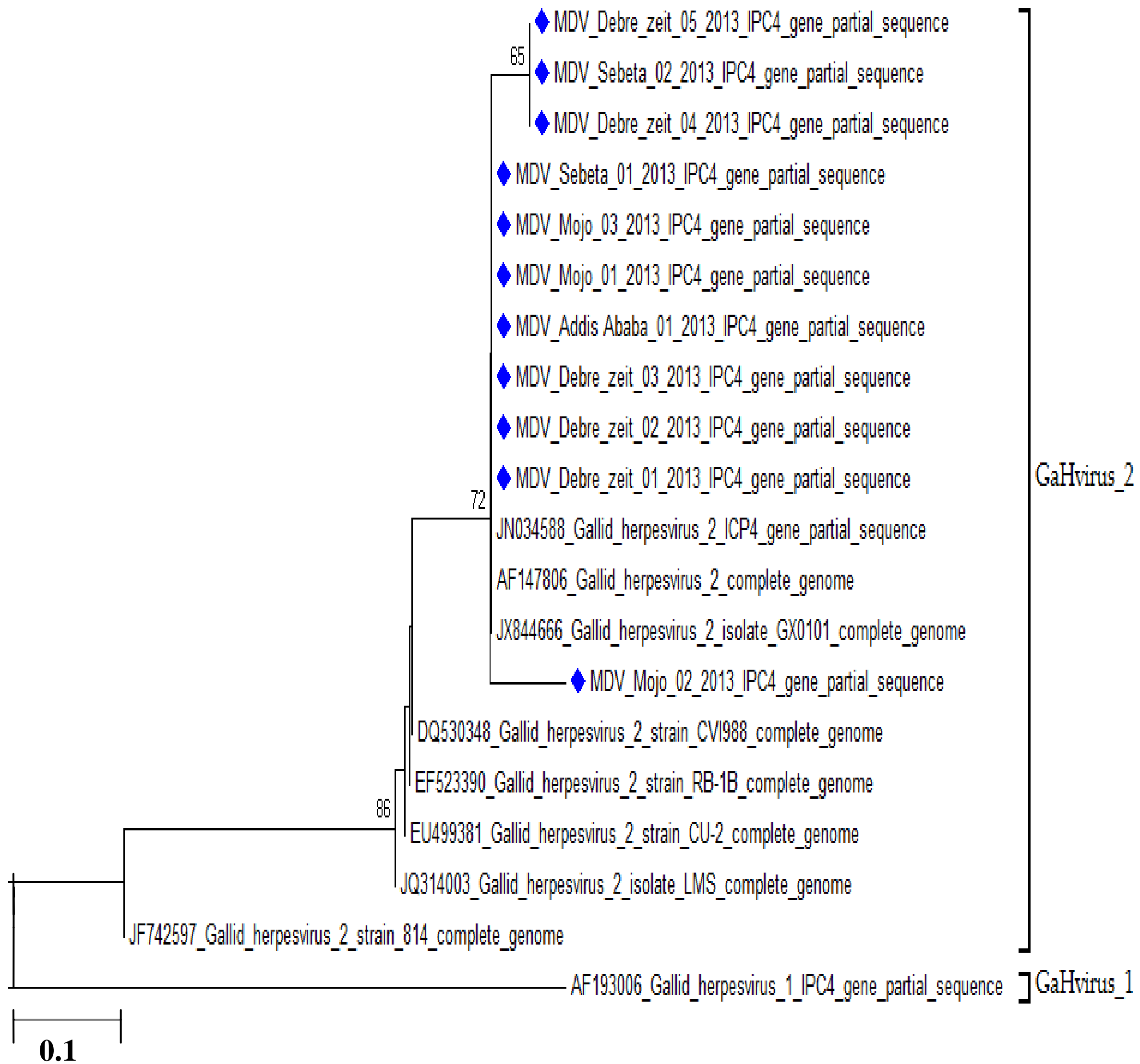

Figure 9. Phylogenetic analysis of 20 Marek's disease viruses including nine retrieved from GenBank. The consensus tree was constructed based on the nucleotide sequence alignments of the ICP4 partial gene sequences using the Neighbor-Joining method and the MEGA5.1 software. The percentages of bootstrap values above 50% (1000 replicates) are indicated on the branches. The homologue ICP4 partial gene sequence from Gallid Herpesvirus-1 (AF193006) isolate retrieved from the GenBank was used as an out-group. The present eleven isolates from Ethiopia are marked with blue colour diamond box.

4.5. Nucleotide variability

Sequence alignment analysis revealed nucleotide variability and similarity among the analysed samples and reference strains (Figure 10). Ethiopian isolates are almost similar with few mismatches compared with reference sequences of Gallid herpesvirus 2 strains. While sequences of Gallid herpesvirus 1 ICP4 gene partial sequence has many nucleotide mismatches compared with Ethiopian MD virus isolates.

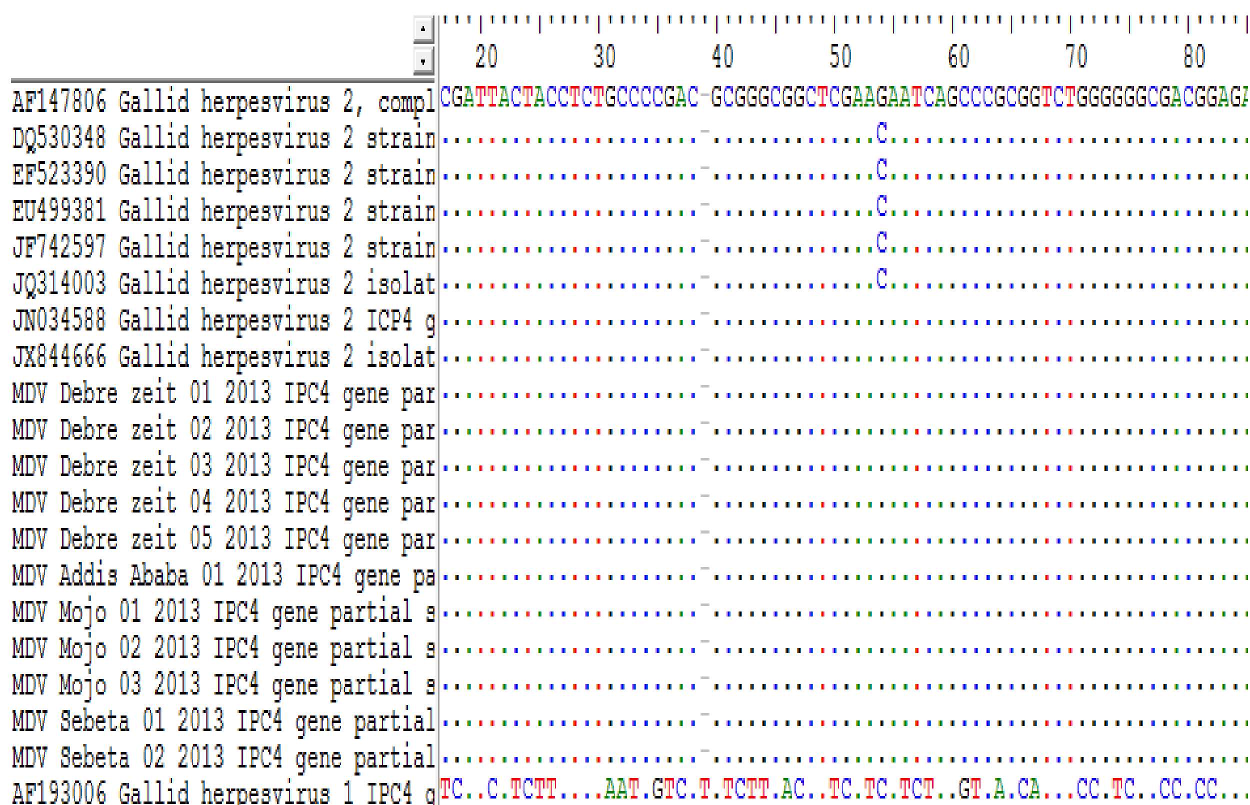

Figure 10. Nucleotide sequence alignment result of the partial ICP4 gene (n=20) revealed the existence of nucleotides similarities and differences among the analysed outbreak and reference sequences. Nucleotides identical with the first sequence are indicated with dots. Nucleotide differences (mismatch) are marked in letter. Hyphen stand for nucleotide absent.

5. DISCUSSION

This study demonstrated the isolation and molecular characterization of marek's disease virus from clinically diseased Ethiopian chickens reared under different production system. The virus was indeed confirmed by using the recommended diagnostic techniques (OIE, 2010) of cell culture, polymerase chain reaction and sequencing of isolates.

Few reports are available on the existence of MD virus in the commercial poultry farms and on its serious health and production impact of the disease in the poultry industry in Ethiopia (Lobago and Woldemeskel, 2004; Duguma *et al.*, 2005). Nevertheless, all reports were not supported by virus isolation and molecular analysis data. From the last couple of years farm owners and professionals has been continuously reporting the suspicion of occurrence of marek's disease virus infection in the poultry farms reared in different geographical areas of the country under different production systems to the National Veterinary Institute despite utilization of the imported vaccine.

Based on the customers disease report the present study was designed to confirm the circulation of marek's disease virus among the chicken population using the recommended diagnostic laboratory techniques. The isolation of the virus on chicken embryo fibroblast cell culture and revealing characteristic marek's disease virus cytopathic effect of rounding of cells and formation of small plaques after third blind passages is in agreement with Delaney *et al*. (1998) and Tan *et al*. (2008) who stated that MD virus was isolated using chicken embryo fibroblast primary cell culture from most samples collected from clinically diseased chickens.

The PCR analysis successfully amplified the virus gene and revealed that the chickens were infected with marek's disease virus by producing the expected band size on agarose gel electrophoresis analysis. This PCR finding is in agreement with the previous report of Kalyani (2010) that the same PCR product band size obtained using the amplification primers M 1.1 and M 1.8 (forward primer M1.1 5'-GGATCGCCCACCACGATTACTACC-3' and reverse primer M1.8 5'-ACTGCCTCACACAACCTCATCTCC-3'). The number of tested samples by

conventional PCR finding is in line with Tan *et al*. (2008) that most of the tested samples were positive for marek's disease virus.

In the present study the sequence analysis using the different bioinformatics software and applications revealed the existence and circulation of marek's disease virus in Ethiopian chicken population.

Genetic relatedness of the present Ethiopian marek's disease virus isolates sequences were computed phylogenetically with the different marek's disease virus reference strains retrieved from the Genbank using MEGA5.1 (Tamura *et al*., 2011). Accordingly, all Ethiopian marek's disease virus isolates were clustered under Gallid Herpes virus type 2 and matched almost perfectly with few nucleotide mismatches with the published MD virus sequences of JN34588, AF147806, JX844666, and others. The nucleotide sequences of the Ethiopian MD virus isolates have many nucleotide mismatches and gaps when compared with nucleotide sequences of Gallid herpesvirus 1 ICP4 gene partial sequence (AF193006). There is a nucleotide/amino acid sequence variations (mismatches and deletion) among the three serotypes, and one of the possible reasons for the variation within serotypes might be due to the adaptation of the virus to the different animal host cell during infection, Nevertheless, there are also nucleotide similarity areas in some of the homologue genes where they have similar functions.

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 (INDOVAX PLC). Each dose of vaccine contains $\geq 10^3$ PFU of HVT FC-126 marek's disease virus strain. The vaccine strain is Meleagrid Herpesvirus 1, and this strain belongs to MDV serotype 3 or Herpes virus of turkeys and is a recommended vaccine strain since it is avirulent and non-oncogenic strain which is more safe to birds and induces antibody production which can protect infection from other serotypes. 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. The present laboratory based research findings will contribute more information to the scientific communities on the

existence of the marek’s disease in Ethiopia. Some of the limitations of this study are the outbreak samples were collected from central Ethiopia on few poultry farms and the PCR assay was targeted on small fragment of the gene; since sequencing of two or more genes and/or bigger fragment will produce more information about the nature of the virus. Full genome sequencing of the present isolates will enrich the information about the virus serotype and pathotype.

To our knowledge the present study on virus isolation, molecular characterization and phylogenetic tree analysis of marek’s disease virus isolates is the first attempt conducted in the country and confirmed the circulation of marek’s disease virus in Ethiopian chickens.

6. CONCLUSION AND RECOMMENDATIONS

Marek's disease is a highly contagious lymphoproliferative viral disease of chickens and is becoming a serious health problem of the poultry industry in Ethiopia. MD is believed to be introduced with the importation of exotic breeds of eggs and day-old chicks and has been reported to be a potential threat to both local and exotic breeds in backyard and commercial farming systems. The present study conducted on pathological samples collected from different geographical areas revealed the circulation of marek's disease virus in Ethiopian chickens and clustered under Gallid Herpes virus type 2. Understanding the molecular epidemiology of MD viruses in the backyard and commercial poultry farms in the present study is highly important for the design of preventive measures and for the development and production of effective vaccine for the control of the spread of the virus. Thus poultry farm owners, professionals and the veterinary service in the Ministry of Agriculture should give more attention on the prevention and control of marek's disease.

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

- Further study on the isolation and molecular characterization of marek's disease virus should be implemented targeting different breeds, geographical areas on back yard and commercial poultry farms.
- Study on the relationship between poultry breeds (local and exotic) with virulence of marek's disease virus should be conducted.
- Strict bio-security measures should be implemented during importation of eggs and day-old chicks.
- Development and production of effective marek's disease vaccine should be facilitated in the country which could protect the chickens from the circulating virus type.

7. REFERENCES

Abdul-Careem M.F., Hunter B.D., Sarson A.J., Mayameei A., Zhou H., and Sharif S. (2006): Marek's disease virus-induced transient paralysis is associated with cytokine gene expression in the nervous system. *Viral Immunology*, **19**(2):167–76.

Afonso C.L., Tulman E.R., Lu Z., Zsak L., Rock D.L., and Kutish G.F. (2001): The genome of turkey herpesvirus. *Journal of Virology*, **75**:971-978.

Anderson A. S., Francesconi A. and Morgan R.W. (1992): Complete nucleotide-sequence of the Marek's-disease virus-ICP4 gene. *Virology* **189:**657-667.

Angamuthu R.S., Baskaran D.R., Gopal J., Devarajan J. and Kathaperumal.K. (2012). Rapid detection of the Marek's disease viral genome in chicken feathers by loop-mediated isothermal amplification. *Journal of Clinical Microbiology*, **50**:961-965.

Arulmozhi A., Saravanan S., Mohan B., and Balasubramaniam G.A.(2011): Marek's disease in vaccinated poultry flocks in and around Namakkal region of Tamil Nadu. *Indian Journal of Vetrinary Pathology*, **35**:45-47.

Ashenafi H. (2000). Survey of Identification of Major Diseases of local chickens in three-selected agro climatic zones in central Ethiopia. DVM thesis, Addis Ababa University, Faculty of Veterinary Medicine, Debre Zeit, Ethiopia, Pp : 1-6.

Baigent S.J., Petherbridge L.J., Howes K., Smith L.P., Currie R.J.W. and Nair V.K: (2005). Absolute quantitation of Marek's disease virus genome copy number in chicken feather and lymphocyte samples using real-time PCR, *Journal of Virology Methods*, **123**:53-64.

Barrow A. and Venugopal K. (1999): Molecular characteristics of very virulent European MDV isolates. *Acta Virologica,* **43**:90-93.

Barrow, A.D., Burgess, S.C., and Venugopal, K. (2001). Invasion of avian macrophages by highly virulent Marek's disease virus strain c12/130 represents a "tropic shift" in the

pathogenesis. In: Schat KA, Morgan RW, Parcells MS, Spencer JL, editors. Proceedings of the 6th international symposium on Marek's disease. Pp: 63–67.

Barrow A., Burgess S.C., Baigent S.J., Howes K. and Nair V.K. (2003): Infection of macrophages by a lymphotropic herpesvirus: a newtropism for Marek's Disease virus. *Journal of General Virology*, **84**:2635–2645.

Becker Y., Asher Y., Tabor E., Davidson I., Malkinson M. and Weisman Y. (1992): Polymerase chain reaction for differentiation between pathogenic and non-pathogenic serotype 1 Marek's Disease Viruses(MDV) and vaccine viruses of MDV-serotypes 2 and 3. *Journal of Virology Methods*, **40**:307-322.

Bemnet G., Ameha Y., Alemayehu Z., Jemanesh K. and Tekalign T. (2003): Fertilizer N effects on yield and grain quality of durum wheat. *Tropical Agriculture (Trinidad)*, **80**(2):1-6.

Biggs P.M. (1967). Studies on Mrek's disease. I. Experimental transmission. *Journal of National Cancer Institute,* **39**:267-280.

Bineesh P.P., Kansal S.K., Banga H.S., Sharma S. and Brar R.S. (2007). Prevalence and pathology of concurrent lung affections in the lymphoproliferative disorders of chicken. *Indian Vetrinary Journal*, **84**:911-914.

Brunovskis P. and Velicer L. (1995): The *Marek's disease virus* (*MDV*) unique short region: *alphaherpesvirus*-homologous, fowl pox virus homologous, and *MDV*-specific genes. *Virology*, **206**:324-338.

Burgess S.C., Young J.R., Baaten B.J., Hunt L., Ross L.N. and Parcells M.S. (2004): Marek's disease is a natural model for lymphomas over expressing, Hodgkin's diseaseantigen (CD30). *Procedure National Acadamic Science USA*, **101**(38):13879–84.

Calnek B.W., Adldinger H.K. and Kahn D.E. (1970): Feather follicle epithelium-a source of enveloped and infectious cell-free herpesvirus from Marek's disease. *Avian Diseases,* **14**: 219-233

Calnek B.W., Schat K.A., Ross L.J.N., Shek W.R. and Chen C-L.H. (1984): Further characterization of Marek's disease virus-infected lymphocytes. I. *In vivo* infection. *International Journal of Cancer,* **33**: 389-395.

Calnek B.W. (1986). Marek's disease - a model for herpesvirus oncology. *Critical Reviews in Microbiology,* **12**: 293-320.

Calnek B.W., Harris R.W., Buscaglia C., Schat K.A. and Lucio, B. (1998): Relationship between the immunosuppressive potential and the pathotype of Marek's disease virus isolates. *Avian Disease*, **42**(1):124–132.

Cantello J.L., Anderson A.S., Morgan R.W. (1994): Identification of latency associated transcripts that map antisense to the ICP4 homolog gene of Marek's disease virus. *Journal of Virology*, **68**: 6280-6290.

Cebrian J., Kaschkadierich C., Berthelot N., and Sheldrick P. (1982): Inverted repeat nucleotide-sequences in the genomes of Marek's disease virus and the herpesvirus of the turkey. *Proceedings of the National Academy of Sciences of the United States of America-Biological Sciences*,**79**:555-558.

Central Statistical Agency. (2009). Agricultural sample survey Vol. II. Statistical Bulletin No. 446. CSA, Addis Ababa, Ethiopia.

Chuahan H.V. and Roy S.Y. (1998): Poultry Disease Diagnosis, Prevention and Control. 7th Edition. W.B. Saunders, India, Pp: 58-429.

Churchill A.E. and Biggs P.M.(1967): Agent of Marek's disease in tissue culture. *Nature,* **215**: 528 - 530.

Cui Z., Lee L,F., Liu J.L. and Kung H.J. (1991). Structural analysis and transcriptional mapping of the Marek's disease virus gene encoding pp38, an antigen associated with transformed cells. *Journal of Virology,* **65**:6509–6515.

Dawit L., Addis M. and Gari G. (2012): Distribution, seasonality and abundance of Sotoxys flies in selected districts of central Ethiopia. *World Applied Science,* **19**(7): 998-1002.

De Laney D.B., Morrow C.J., Read K.M. and Tannock G.A. (1998). The development and evaluation of two tissues culture grown Marek's disease challenge viruses. *Avian Pathology*, **27**:472- 477.

Dessie T. (1996). Studies on village poultry production systems in the central highlands of Ethiopia, MSc thesis, department of animal nutrition and management. Swedish, University of Agricultural Sciences.

Dessie T. and Ogle B. (2001): Village poultry production systems in the central highlands of Ethiopia. *Tropical Animal Health and Production*, **33**: 521-537.

Duguma R., Yami A., Dana N., Hassen F. and Esatu W. (2005): Marek's disease in local chicken strains of Ethiopia reared under confined management regime in central Ethiopia. *Revue de Médecine Vétérinaire*, **156**(11): 541-546.

Duguma R., Dana N. and Yami A. (2006): Marek's Disease Vaccination Opened the Door to Rear Indigenous Chickens of Ethiopia Under Confined Management. *International Journal of Applied Research in Veterinary Medicine*, **4** (2): 121-127.

Fukuchi K., Sudo M., Lee Y.A., Tanaka A. and Nonoyama M. (1984): Structure of Marek's-disease virus-DNA - Detailed restriction enzyme map. *Journal of Virology*, **51**:102-109.

Gimeno I.M., Witter R.L., Hunt H.D., Lee L.F., Reddy S.M. and Neumann U. (2001): Chronological study of brain alterations induced by a very virulent plus (vv+) strain of Marek's disease virus (MDV). In: Schat ,K.A., Morgan,R.W, Parcells M.S., Spencer, J.L, editors. Proceedings of the 6th international symposium on Marek's disease. Pp: 21-6.

Gimeno I.M., Witter R.L. and Neumann U. (2002): Neuropathotyping: a new system to classify Marek's disease virus. *Avian Disease*, **46**(4):909-918.

Gimeno I.M. (2004). Future strategies for controlling Marek´s disease. In Marek´s disease: an evolving disease, Davison F, Nair V (eds.). Elsevier Academic Press, London Pp. 186-199.

Gimeno I.M. and Silva R.F. (2006): Pathogenesis of MDV in the eye: neoplasia or autoimmunity? In: Parcells MS, Morgan RW, Burnside J, et al., editors. Proceedings of the 4th International Workshop on the molecular pathogenesis of Marek's disease. Pp: 16.

Gimeno I.M. (2008). Marek's disease vaccines: a solution for today but a worry for tomorrow? *Vaccine,* **18**:26.

Goto M., Honda E., Ogura A., Nomoto A. and Hanaki K. (2009): Colorimetric detection of loop-mediated isothermal amplification reaction by using hydroxy naphthol blue. *Biotechniques*, **46**:167-172.

Gopal S., Manoharan P., Kathaperumal K., Chidambaram B. and Divya K.C. (2012): Differential detection of avian oncogenic viruses in poultry layer farms and turkeys by use of multiplex PCR. *Journal of Clinical Microbiology*, **50**: 2668-2673.

Graham P.H. (1976). Prevention of Marek's disease: A review. *Cancer Research*, **36**: 696-700.

Hall TA. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series*, **41**:95–8.

Handberg K. J., Nielsen O. L., Jergensen P. H. (2001): The use of serotype 1 and serotype 3 - specific polymerase chain reaction for the detection of Marek's disease virus in chickens. *Avian Pathology*, **30**: 243-249.

Hennig H., Osterrieder N., Muller-Steinhardt M., Teichert H.M., Kirchner H. and Wandinger K.P. (2003): Detection of Marek's disease virus DNA in chicken but not in human plasma. *Journal of Clinical Microbiology*, **41**(6): 2428- 2432.

Hunt H., Lupiani B., Miller M., Gimeno I., Lee L.F. and Parcells M.S. (2001): Marek's disease virus down-regulates surface expression of MHC (B Complex) Class I (BF) glycoproteins during active but not latent infection of chicken cells**.** *Virology*, **282**:198-205.

International Committee on Taxonomy of Virus, 2011. Virus Taxonomy. (2011). Release (current) .http:// ictvonline. org/virus Taxonomy.asp? version = 2011& bhcp=1.

Islam A., Harrision B., Cheetham B.F., Mahony T.J., Young P.L. and Walkden-Brown S.W. (2004): Differential amplification and quantitation of Marek's Disease viruses using real-time polymerase chain reaction. *Journal of Virology Method*, **119**: 103-13.

Islam A., Cheetham B.F., Mahony T.J., Young P.L. and Walkden-Brown S.W. (2006): Absolute quantitation of Marek's disease virus and herpesvirus of turkeys in chicken lymphocyte, feather tip and dust samples using real-time PCR. *Journal of Virology Methods,* **132**:127-134.

Izumiya Y., Jang H., Ono M. and Mikami T. (2001): A complete genomic DNA sequence of *Marek's disease virus* type 2, strain HPRS24. *Current Topic Microbiology Immunology*, **255**: 191-221.

Jadhav M.M., Shelar P.D., Patil P.S., Dhumal R.V., Suryawanshi D.S. and Nehete R.S. (2007): Pathological studies in Marek's disease outbreak in layers. *Journal Bombay Vetrinary Collage*, **15**:4-6.

Jarosinski K.W., Yunis R., O'Connell P.H., Markowski-Grimsrud C.J. and Schat K.A. (2002): Influence of genetic resistance of the chicken and virulence of Marek's disease virus (MDV) on nitric oxide responses after MDV infection. *Avian Disease,* **46**:636-649.

Jarosinski K.W., Njaa B.L., O'Connell P.H. and Schat K.A. (2005): Pro-inflammatory responses in chicken spleen and brain tissues after infection with very virulent plus Marek's disease virus. *Viral Immunology*, **18**:148-161.

Jeurissen S.H.M., Janse E.M., Wagenaar F. and de Boer G.F. (1992): The role of splenic ellipsoid-associated reticulum cells in the pathogenesis of Marek's disease. In: Proceedings of the 4th international symposium on Marek's disease, 19th World's Poultry Congress, volume 1. Pp: 211-215.

Jones D., Lee L., Liu J. L., Kung H. J. and Tillotson J. K. (1992): *Procedure National Academy Science USA,* **89**: 4042–4046.

Josipovic D. (1990). Marek's disease - still a problem in poultry. *Laviculture en Mediterranee Ser.* **49**(2): 185-194.

Kalyani I. H., Tajpara M. M., Jhala M. K., Bhanderi B. B., Nayak J. B. and Purohit J. H. (2010): Characterization of the ICP4 gene in pathogenic Marek's disease virus of poultry in Gujarat, India, using PCR and sequencing. *Veterinarski Arhiv Journal*, **80**: 683-692.

Kamaldeep P.C., Sharma N., Jindal G. and Narang G. (2007): Occurrence of marek's disease in vaccinated poultry flocks of Haryana(India). *International Journal Poultry Scinece*, **6**: 372-377.

Kataria J.M., Madan M.C., Sohini D., Dash B.B. and Dhama K. (2005): Diagnosis and immunoprophylaxis of economically important poultry disease: A review. *Indian Iournal of Animal Science*, **75**: 555-567.

Kaupp B.F. (1921). Paralysis of the domestic fowl. *Journal of American Association Instructors Investigators Poultry Husbandry*, **7**: 25-31.

Kingham B.F., Zelnick V., Kopacek J., Ney E. and Schmidt C. (2001): The genome of herpesvirus of turkeys: comparative analysis with Marek's disease viruses. *Journal of General Virology*, **82**: 1123-1135.

Kreager K.S. (1998). Chicken industry strategies for control of tumor virus infections. *Poultry Science*, **77**: 1213-1216.

Kuria J.K., Wahome R.G. and Gitao P.K. (2001): An account of a recent outbreak of Marek's disease in Kenya. *Kenya Vetrinary*, **21**: 34-36.

Lee S.I., Ohashi K., Morimura T., Sugimoto C. and Onuma M. (1999): Re-isolation of Marek's disease virus from T cell subsets of vaccinated and non-vaccinated chickens. *Archives of Virology*, **144**:45 - 54.

Lee S.I., Takagi M., Ohashi K., Sugimoto C. and Onuma M. (2000): Difference in the meq gene between oncogenic and attenuated strains of Marek's disease virus serotype 1. *Journal of Veterinary Medicine Science*, **62**:287-292.

Lobago F. and Woldemeskel M. (2004): An outbreak of Marek's disease in chickens in central Ethiopia. *Tropical Animal Health and Protection*, **36** (4): 397-406.

Marek J. (1907). Multiple Nervenentzuendung (Polyneuritis) bei Huehnern. *Deutsche Tieraerztliche Wochenschrift,* **15**:417-421.

McKie E. A., Ubukata E., Hasegawa S., Zhang S., Nonoyama M. and Tanaka A. (1995): The transcripts from the sequences flanking the short component of Marek's disease virus during latent infection form a unique family of 39-coterminal RNAs. *Journal of Virology*, **9**: 1310- 1314.

Morgan R., Anderson A., Bernberg E., Kamboj S., Huang E., Lagasse G., Isaacs G., Parcells M., Meyers B.C., Green P.J. and Burnside J. (2008): Sequence conservation and differential expression of Marek's Disease Virus MicroRNAs. *Journal of Virology*, **82**(24): 12213-12220.

Morimura T., Ohashi K., Kon Y., Hattori M., Sugimoto C. and Onuma M. (1996): Apoptosis and CD8-down-regulation in the thymus of chickens infected with Marek's disease virus. *Arch Virology*, **141**:2243-2249.

Murata S., Chang K.S., Lee S.I., Konnai S., Onuma M. and Ohashi K.(2007): Developement of a nested polymerase chain reaction method to detect oncogenic Marek's disease virus from feather tips. *Journal of Veterinary Diagnostic Investigation*, **19**:471-478.

Murphy F.A., Gibbs E.P.J., Hozinek M.C. and Studdert M.J. (1999): Veterinary Virolology, 3rd Edition. London: Academic press, Pp: 405-409.

Nazerian K., Solomon J.J., Witter R.L. and Burmester B.R.(1968): Studies on the etiology of Marek's disease . II. Finding of a herpesvirus in cell culture. *Procedure Soc. Exp. Biology Medicine,* **127**: 177-172.

Notomi T., Okayama H., Masubuchi H., Yonekawa T., Watanabe K., Amino N. and Hase T. (2000): Loop-mediated isothermal amplification of DNA. *Nucleic Acids Research*, **28**: 63.

OIE (2010). Marek's Disease.OIE Manual of diagnostic tests and vaccines of terrestrial animals. Office International des Epizootics (OIE), Paris, France. pp. 1-11.

Okazaki W., Purchase H.G. and Burmester B.R. (1970): Protection against Marek's disease by vaccination with a herpesvirus of turkey. *Avian Disease*, **14**: 413-429.

Okwor E.C. and Eze D.C. (2011): Outbreaks and persistence of marek's disease ib batches of birds reared in a poultry farm located in Nsukka,South East Nigeria. *International Journal of Poultry Science*, **10**: 617-620.

Pappenheimer A.M., Dunn L.C. and Vone V. (1926): A study of fowl paralysis (neuro-lymphomatosis gallinarum). *Storrs Agriculture Experimental Static Bulletin*, **143**:187-290.

Payne L.N. and Venugopal N. (2000): Neoplastic diseases: Marek's disease, avian leukosis and reticuloendotheliosis. *Revue scientifique et technique,* **19**(2): 544-564.

Phillips P.A. and Biggs P.M. (1972): Course of infection in tissue of susceptible chickens after exposure to strains of Marek’s disease and turkey herpesvirus. *Journal of National Cancer Institute*, **49**: 1367–1373.

Powell P.C. and Lombardini F. (1986): Isolation of very virulent path types of Marek's disease virus from vaccinated chickens in Europe. *Vetrinary Record*, **118**: 688-691.

Pratt W. D., Cantello J., Morgan R.W. and Schat K.A. (1994): Enhanced expression of the Marek’s disease virus-specific phosphoproteins pp38/41 after stable transfection of MSB-1 cells with the Marek’s disease virus homolog of ICP4. *Virology*, **201**: 132-136.

Purchase H.G., Okazaki W. and Burmester B.R. (1971): Field trials with the herpesvirus of turkey (HVT) strain FC126 as a vaccine against Marek's disease. *Poultry science*, **50**: 775 -783.

Raja A., Dhinakar R.G., Bhuvaneswari P., Balachandran C. and Kumanan K. (2009): Detection of virulent MD virus in poultry in India. *Acta Virology*, **53**: 255-260.

Rajkhowa T.K. (2005). Acute Marek's Disease. In:Diseases of Poultry, Saif, Y.M., Fadly,A.M., Glisson, J.R., McDougald, L.R., Nolan, L.K., and Swayne, D.E.(Eds.) 12th Edn, Blackwell Pulishing, Iowa, Pp: 452-512.

Renz K.G., Islam A., Cheetham B.F. and Walkden-Brown S.W (2006): Absolute quantification using real-time polymerase chain reaction of Marek’s disease virus serotype 2 in field dust samples, feather tips and spleens. *Journal of Virology Methods,* **135**: 186-191.

Rispens B.H., van Vloten H., Mastenbroek N., Mass H.J.L. and Schat K.A. (1972): Control of Marek's disease in the Netherlands.1. Isolation of an avirulent Marek's disease virus (Strain CVI-988) and its use in laboratory vaccination trials. *Avian Disease*, **16**: 108-125.

Rodriguez A., Vigorito E., Clare S., Warren M.V., Couttet P., Soond D.R., Van Dongen S., Grocock R.J., Das P., Miska E.A., Vetrie D., Okkenhaug K., Enright A.J., Dougan G., Turner M. and Bradley A. (2007): Requirement of bic/microRNA-155 for normal immune function. *Science,* **316**: 608-611.

Ross L.J.N.(1998). Recombinant vaccines against Marek'sdisease. *Avian Pathology*, **27**(1): 65-73.

Ryan K.J. and Ray C.G. (editors) (2004): Sherris Medical Microbiology (4th edition). McGraw Hill. ISBN o-8384-8529-9.

Sandri-Goldin, R.M. (editor). (2006). Alpha Herpesviruses: Molecular and Cellular Biology. Caister Academic Press . ISBN 978-904455-09-7.

Schat K.A. and Calnek B.W. (1978): Characterization of an apparently nononcogenic Marek's disease virus. *Journal of National Cancer Institute*, **60**: 1075-1082.

Schat K. A. and Purchase H.G.(1998): Cell-culture methods. In: Swayne, D. E. , Glisson, J. R., Jackwood, M. W., Pearson, J. E. , and Reed W. M.(Eds.) A laboratory manual for the isolation and identification of avian pathogens, 4th Edition. American Association of Avian Pathologists, Kennett Square, Pennsylvania, Pp: 223-234.

Schat K.A. and Nair V. (2008): Marek’s disease. In: Saif Y.M. *et al*., (Eds.) Diseases of Poultry. 12th Edition. Blackwell Publishing, Ames Iowa, USA, Pp: 452–514.

Shambhu D.S., Rajamani B., Asok K., Rajib D., Amit K.V. and Kuldeep D. (2012): Recent Trends in Diagnosis and Control of Marek's Diease (MD) in poultry. *Pakistan Journal of Biological Sciences*, **15**(20): 964-970.

Shek W.R., Schat K.A. and Calnek B.W. (1982): Characterization of non-oncogenic Marek's disease virus-infected and turkey herpesvirus infected lymphocytes. *Journal of General Virology*, **63**:333–41.

Shek W, Calnek B, Schat K, Chen C. (1983): Characterization of Marek's disease virus-infected lymphocytes: discrimination between cytolytically and latently infected cells. *Journal of National Cancer Inst*itute, **70**:485-491.

Silva R.F., Reddy S.M. and Lupiani B. (2004): Expansion of a unique region in the Marek's disease virus genome occurs concomitantly with attenuation but is not sufficient to cause attenuation. *Journal Virology*, **78**(2): 733-740.

Spatz S. J., Petherbridge L., Zhao Y.G. and Nair V.(2007): Comparative full-length sequence analysis of oncogenic and vaccine (Rispens) strains of Marek's disease virus. *Journal of General Virology*, **88:**1080-1096.

Tamura K., Peterson D., Peterson N., Stecher G., Nei M. and Kumar S. (2011): MEGA5 molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology Evolution,* **28**: 2731-2739.

Tan J., Cooke J., Clarke N. and Tannock G.A. (2008): Optimization of methods for the isolation of Marek's disease viruses in primary chicken cell cultures. *Journal of Virology Methods*, **147**:312–318.

Tesfaye A. (2009). Steady-state ground water flow and containant transport modelling of Akaki well field and its surrounding catchment. M.Sc thesis submitted to the International Institute for geo-information science and earth observation, The Netherlands.

Thompson J.D., Higgins D.G., Gibson T.J. and Clustal W. (1994): improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research,* **22**:4673–80.

Tulman E.R., Afonso C.L., Lu Z., Zsak L., Rock D.L .and Kutish G.F. (2000): The genome of a very virulent Marek's disease virus. *Journal of Virology,* **74**:7980–7988.

Venugopal K. and Payne L.N. (1995): Molecular pathogenesis of Marek's disease - recent developments. *Avian Pathology,* **24**:597-609.

Venugopal K., Jones R.C. and Gough R. (2001): *Herpesviridae*. In: Jordan F., Pattison M., Alexander D. and Faragher T. (Eds.). Poultry disease, 5th Edition. London: W.B. Saunders, Pp: 221-234.

Venugopal N. (2005). Evolution of Marek's disease - A paradigm for incessant race between the pathogen and the host. *The Veterinary Journal*, **170**: 175-183.

Viral zone. http://viralzone expasy.org/all_by_species/522.html#tab6. Accessed on 29 May 2014.

Walkden-Brown S.W., Islam A., Islam A.F., Burgess S.K., Groves P.J. and Cooke J. (2013): Pathotyping of Australian isolates of Marek's disease virus in commercial broiler chickens vaccinated with herpesvirus of turkeys (HVT) or bivalent (HVT/SB1) vaccine and association with viral load in the spleen and feather dander. *Australian Veterinary Journal*, **91**: 341-350.

Wei X., Shi X., Zhao Y., Zhang J. and Wang M. (2012): Development of a rapid and specific loop-mediated isothermal amplification detection method that targets Marek's disease virus meq gene. *Journal of Virology Methods*, **77**: 1197-1203.

Witter R.L., Nazerian K., Purchase H. G. and Burgoyne G.H. (1970): Isolation from turkeys of a cell-associated herpesvirus antigenically related to Marek's disease virus. *American Journal of Veterinary Research,* **31**: 525-538.

Witter R. L. (1992). Influence of serotype and virus strain on synergism between Marek's disease vaccine viruses. *Avian Pathology, **21***:601–614.

Witter R. L. (1997). Increased virulence of Marek's disease virus field isolates. *Avian Disease*, **41**:149–163.

Witter R.L. and Schat K.A. (2003): Marek´s disease. In Diseases of Poultry Saif Y.M., Barnes H.J., Glisson J.R., Fadly A.M., McDougald L.R. and Swayne D.E. (Eds.). Diseases of Poultry. Iowa State University Press, Ames, IA , Pp: 407-464.

Witter R. L., Gimeno I. M., Pandiri A. R. and Fadly A. M. (2010): The differential diagnosis of lymphoid and myeloid tumors in the chicken. In: Tumor diagnosis manual. American Association of Avian Pathologists, Pp: 1-84.

World Organization of Animal Health (Office International des Epizootics), World Animal Health Information Database. July-December 2011, *Retrieved on 05 May 2014* http://www.oie.int/wahis_2/public/wahid.php/Diseaseinformation/Diseasedistributionmap

Wozniakowski G., Samorek-Salamonowicz E. and Kozdrun W. (2011): Rapid detection of Marek's disease virus in feather follicles by loop-mediated amplification. *Avian Disease* , **55**: 462-467.

Xie Q., Anderson A.S. and Morgan R.W. (1996). Marek's disease virus (MDV) ICP4, pp38, and meq genes are involved in the maintenance of transformation of MDCC-MSB1 MDV-transformed lymphoblastoid cells. *Journal of Virology,* **70**:1125-1131.

Xing Z. and Schat K.A. (2000): Expression of cytokine genes in Marek's disease virus-infected chickens and chicken embryo fibroblast cultures. *Immunology,* **100**:70-76.

Xing Z. and Schat K. A. (2000): Inhibitory effects of nitric oxide and gamma interferon on in vitro and in vivo replication of Marek's disease virus. *Journal of Virology,* **74**:3605-3612.

Yami A. (1995). Poultry production in Ethiopia. *World Poultry Science Journal*, **51**: 197-201.

Zander D.V., Hill R.W., Raymond R.G., Balch R.K., Mitchell R.W. and Dunsing J.W. (1972): The use of blood from selected chickens as an immunizing agent for Marek's disease. *Avian Disease*, **16**: 163-178.

Zeleke A., Gelaye E., Sori T., Ayelet G., Sirak A., Zekarias B. (2005a): Investigation on infectious bursal disease outbreak in Debre Zeit. *International Journal of Poultry Science*, **7**: 504-506.

Zeleke A., Sori T., Gelaye E. and Ayelet G. (2005b): Newcastle disease in village chickens in the southern and rift valley districts in Ethiopia. *International Journal of Poultry Science*, **7**: 508-510.

Zhu G.S., Ojima T., Hironaka T., Ihara T. and Mizukoshi N. (1992): Differentiation of oncogenic and strains of Marek's disease virus type 1 by using polymerase chain reaction DNA amplification. *Avian Disease*, **36**: 637-645.

8. APPENDICES

Appendix 1. Preparation of chicken embryo fibroblast (CEF) cell cultures

Use 9-11 day old embryos.

1. Spray eggs with Bioguard disinfectant place in hood. Using sterile technique, open shell and remove embryo with blunt ended curved forceps. Place all media and trypsin in 37°C water bath
2. Place embryos in petridish and cut off heads. Removal of limbs and viscera is optional.
3. Transfer bodies to new petridish or beaker containing PBS without calcium or magnesium. In the beaker, the bodies can be fragmented by carefully chopping them with sterile scissors.
4. Wash with PBS 3-4 times to remove red blood cells.
5. Pour tissue fragments into trypsinization flask containing magnetic stirring bar. Add about 50 ml pre-warmed (37°C) trypsin solution (0.25%) to flask and put on stir plate at slow speed into 37°C incubator for 10-15 minutes. Pour off supernatant into centrifuge tube with calf serum. Add 50 ml trypsin solution and stir slowly in 37°C incubator for 8 minutes. (Total trypsinization time: 30-35 minutes at 37°C.) This may be repeated 1 more time for a total of 3 trypsinizations.
6. Centrifuge 10 min. at 1000 rpm. Note the amount of pelleted cells obtained. Pour off trypsin solution and resuspend cells in 3-5 ml GMEM. The cells may be counted or diluted 1:200.

Appendix 2. Growth and maintenance media

Growth medium consist 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 supplement with only 2-5% FBS.

Appendix 3. DNA extraction

Protocol 1: DNA extraction from tissue sample (Qiagen, Germany)

- Cut tissue (≤ 10mg spleen or ≤ 25mg other tissue) into small pieces, and place in a 1.5 ml microcentrifuge tube. Add 180 µl Buffer ATL. Add 20 µl proteinase K, mix by vortexing, and incubate at 56°C until completely lysed. Vortex occasionally during incubation. Vortex 15 s directly before proceeding to step 2.
- Add 200 µl Buffer AL. Mix thoroughly by vortexing. Incubate samples at 56°C for 10 min.
- Add 200 µl ethanol (96-100%). Mix thoroughly by vortexing.
- 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 min. Discard the flow-through and collection tube.
- Place the spin column in a new 2 ml collection tube. Add 500 µl Buffer AW1. Centrifuge for 1 min at ≥6000 x g. Discard the flow-through and collection tube.
- Place the spin column in a new 2 ml collection tube. Add 500 µl Buffer AW2, and centrifuge for 3 min at 20,000 x g (14,000 rpm). Discard the flow-through and collection tube.
- Transfer the spin column to a new 1.5 ml or 2 ml microcentrifuge tube.
- Elute the DNA by adding 50µl Buffer AE to the center of the spin column membrane. Incubate for 1 min at room temperature (15-25°C). Centrifuge for 1 min at ≥ 6000 x g.

Protocol 2: Agarose Gel Electrophorosis of PCR product

1. Prepare 60 ml of 1.5 % agarose in 1x TBE buffer.
2. Either heat in microwave for ~2 min on full powder or place in beaker of being of boiling water until melted.
3. Allow to cool to about 450C and add 2.5 µl /ml red gel.
4. Pour gel and insert well former (comb). Allow to set on a flat surface for about 15 min.
5. Pour buffer 1x TBE into tank and remove comb from gel.
6. Prepare samples in tubes , a multiwell plate or on parafilm .

   1 µl loading buffer

   5 µl PCR product
7. Prepare molecular weight marker.

   0.5 µl ml molecular weight marker VI (Boehringer)

   1 µl loading buffer

   4.5 µlH_2O
8. Load samples into the wells formed in the gel. It is often useful to load the molecular weight markers in both the first and last lanes.
9. Electrophoreses at 100 volts for 20 min (minimum) or 10 volts overnight.
10. View and photograph the gel on an UV- transilluminator. Use UV- safety spectacles.

Appendix 4. 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	1 liter

Sterilized by Autoclave

Appendix 5. Trypsin Solution (0.25%)

NaCl	8.0 g
KCl	0.4 g
Glucose 0.4 g	1.0 ml
Phenol Red (0.5% solution)	1.0 ml
Trypsin (1:250)	1.0 ml
$NaHCO_3$	0.35 g
Purified H_20 q.s.	1 liter

Adjust pH to 7.4 with $NaHCO_3$ solution

Appendix 6. SPGA/ EDTA (sucrose, phosphate, glutamate and albumin/ethylenediamine tetra-acetic acid) buffer preparation

	Concentration(M)	Weight
Sucrose	0.2180M	7.462 g
Monopotassium phospoate	0.0038M	0.052 g
Dipotassium 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		100 ml

pH 6.5