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Isolation and molecular characterization of chicken Marek's disease virus in selected districts of Ethiopia

MSc Thesis

By

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Addis Ababa University, College Of Veterinary Medicine and Agriculture

Department of Microbiology, Immunology and Veterinary Public Health

MSc program in Veterinary Microbiology

June 2017

Bishoftu, Ethiopia

Isolation and molecular characterization of chicken Marek's disease virus in selected districts of Ethiopia

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

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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: Kenenet Atnafu Entitled " Isolation and molecular characterization of chicken Marek's disease virus in selected districts of Ethiopia" 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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STATEMENT OF AUTHOR

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

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LIST OF 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	Revolution 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

ABSTRACT

A cross sectional study was conducted on clinically diseased chickens suspected of Marek's disease (MD) virus infection in a backyard and commercialized poultry farms from November, 2016 to May, 2017 in selected districts of Ethiopia. Clinical and post-mortem examination was conducted on 33 clinically diseased chickens suspected of MD virus infection and thus virus isolation on chicken embryo fibroblast (CEF) cell and molecular and phylogenetic characterization of Marek’s disease virus were conducted. Typical clinical signs of MD were observed in all of the 33 clinically examined chickens. Out of the 10 autopsied chicken gross lesions were observed on 8(80%) chicken on the liver and heart as well as enlargement of spleen were observed. 165 feather follicles and 33 spleen samples were collected from the 33 clinically examined chickens under sterile condition and virus isolation was performed using CEF cell culture. Out of 7 pooled spleen samples, viruses were isolated from 5 (71.4%) preparations and also out of 10 pooled feather samples, viruses were isolated from 9 (90%). All the isolates that were grown and developed CPEs shows visible small plaques on CEF cell cultures starting from the 4th days of 3rd blind passage. Further confirmation of the virus was conducted by conventional polymerase chain reaction using MD virus specific primers targeting to amplify the ICP4 gene and Meq gene on 7 and 5 pooled samples respectively. The expected band sizes of 318bp as well as 600bp were amplified by 6 and 5 of the pooled samples out of 7 and 5 pooled samples respectively. Positive PCR products were further subjected to sequencing using the amplification MDV1OP forward and backward primers and the sequence analysis result using MEGA6 bioinformatics software confirmed that the chickens were infected with MD virus. Phylogenetic tree construction revealed that the current sequenced isolates were clustered together with Gallid herpes virus 2 serotype 1 MDV. Further study on the isolation and molecular characterization of chicken Marek’s disease virus in all regions of the country were recommended.

Key words: *Marek’s disease, Marek’s disease virus, molecular characterization, polymerase chain reaction, sequencing, virus isolation.*

1. INTRODUCTION

In Ethiopia chicken production are the most widespread and almost every rural family owns chickens, which provide a valuable source of family protein and income (Tadelle *et al*., 2003). The total chicken population in the country is estimated to be 56.87 million with native chicken representing 96.9%, hybrid chicken 0.54% and exotic breeds 2.56% (CSA, 2014). The most dominant chicken types reared in Ethiopia are local ecotypes, which show a large variation in body position, plumage color, comb type and productivity (Halima, 2007). The chicken production system in Ethiopia can be characterized by not market oriented, low input, scavenging and traditional management system consisting of local breeds (Alemu and Tadelle, 1997). However, the economic contribution of the sector is not still proportional to the huge chicken numbers (Aberra, 2000; Halima, 2007).

Chicken production 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 threat 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).

Marek's disease is a highly contagious and one of the most economically devastating oncogenic and/or paralytic viral diseases 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 Schat, 2003). MD was first described in 1907 by a veterinarian named József Marek, after whom the disease was named. The first cases of MD were reported in the US in 1914 (Witter and Schat, 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, 67.9% (340/503):66.2% (333/503) respectively, on indigenous chickens in Ethiopia were nearly equal, indicating that MD is highly fatal (97.9%) to the local breeds. On the other hand MD vaccination significantly increased the survival rate of the local breeds (Duguma *et al*., 2006). This indicates the dramatic effect of MD vaccination on the survival of the indigenous chickens and vaccination could be a vital instrument to combat against the threat by MD.

Previously, MD vaccine was being availed by importation from India (INDOVAX Private Limited). These vaccines were 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). In the year 2013/14, NVI imported 1.8 million doses of MD vaccine (HVT FC-126 strain) and distributed for customers. Imported vaccines are acquired from elsewhere via foreign currency and with higher transportation cost, and this makes them to be more costly. Starting from 2016 NVI inaugurated to produce the vaccine from HVT FC-126 strain of MDV. Nevertheless, still there are a lot of MD outbreak reports in different regions of the country where imported MD vaccines were still in use. 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. Previously there was one study that has been conducted in central part of

Ethiopia (Birhan, 2014) which managed to isolation and sequence MDV serotype 1, Gallid Herpesvirus-2. However, further MD outbreak investigation in different agro-ecological regions of the country is mandatory so that development and production of effective MD vaccine from locally isolated MD virus strains will be possible. Therefore, the objectives of this study are:

- To isolate the locally circulating strains of MD virus in selected districts of Ethiopian.
- To undertake molecular characterization and phylogenetic analysis of MD virus isolates.

2. LITERATURE REVIEW

2.1. History of Marek's Disease

Marek’s disease is a fatal lymphoproliferative disease in domesticated chickens (Gallus domesticus) with its inception in 1907 by a renowned veterinarian Dr. Joseph Marek at department of Royal Hungarian veterinary school in Budapest. Initially the disease was described as ‘polyneuritis’ while studying four adult cockerels, which were affected by paralysis of wings and legs. He noted thickening of sacral plexus and spinal routes which were infiltrated by mononuclear cells (Biggs, 1967). The first report of Marek’s disease in united kingdom was made in a meeting at Royal society of medicine by Galloway in 1929.For another 40 years the etiology, nature of lesions and transmission was argued until period between 1962-1970 when herpesvirus was isolated from turkeys and vaccine being produced (Payne, 1979). Before introduction of vaccination in 1970, Marek’s disease caused annual losses of 150 million USD in United States and 40 million USD in United Kingdom (Murphy *et al.*, 1999).

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). Birds get infected by inhalation of infected dust from the poultry houses, and following a complex life cycle, the virus is shed from the feather follicle of infected birds (Baigent and Davison, 2004). 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. 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. MD results in enlarged nerves and in tumor formation in nerve, organ, muscle and epithelial tissue. 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. Affected birds are more susceptible to other infectious diseases (OIE, 2010).

2.3. Etiology

The causative agent of the diseases is Marek's Disease Virus (MDV) and as per the recent classification by the International Committee on Taxonomy of Viruses (ICTV, 2011), it is placed in Order *Herpesvirales*, family *Herpesviridae*, subfamily *Alphaherpesvirinae*, genus *Mardivirus* (Marek's disease-like viruses) and species *Gallid Herpesvirus 2 (GaHV-2).* 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 subfamily Alphaherpesvirinae, genus Mardivirus, and species Gallid Herpesvirus 2 (GaHV-2) (Schat and Nair, 2008; ICTV, 2011).

2.4. Serotypes

Three serotypes of MDV and related Herpes viruses have been defined: 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, very virulent (vv) MDV, and very virulent plus (vv+) MDV (Witter and Schat, 2003).

New pathotypes have been emerging indicating continuous evolution of MDV towards greater virulence. The increase in virulence of MDV over the past 50 year is of major significance. The shift from mMDV to vMDV strains in the late 1950s, the shift from vMDV to vvMDV in the late 1970s and, more recently, the appearance of the putative vv+ MDV in the early 1990s have each resulted in the potential for greater disease losses that have persisted until introduction of a more effective vaccine. The main basis for the pathotype classification is the ability to cause disease in chickens immunized with increasingly effective vaccines (Witter, 1997).

Table 1: Classification of MDV serotypes and their representative 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,CVI-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) (Herpesvirus of Turkey)

Source: Shambhu *et al*., 2012

2.5. Marek's disease virus

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.

2.5.1. 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.

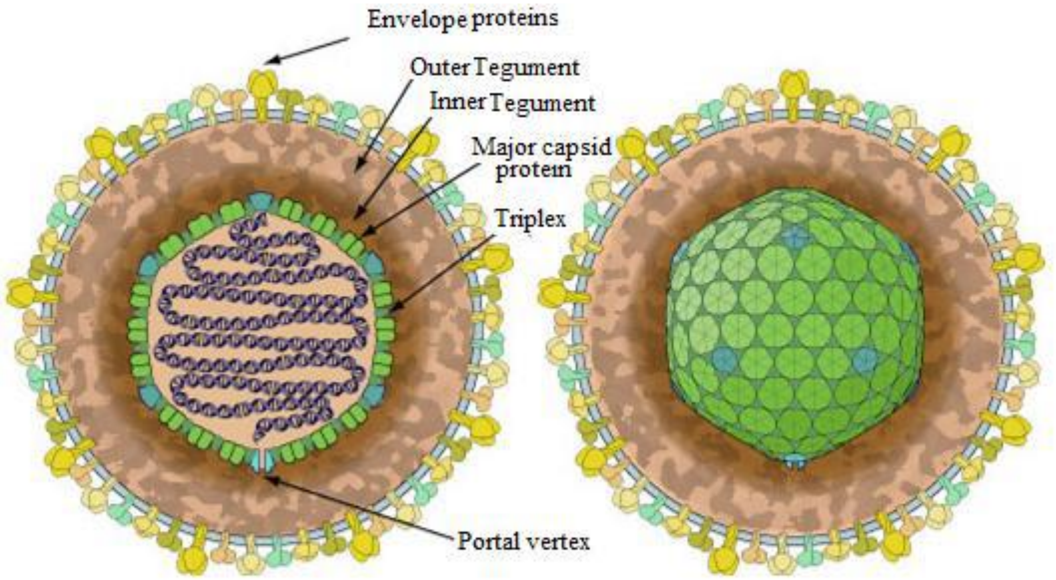

Figure 1: Virion structure of herpesvirus (Source: Viral zone, 2012).

2.5.2. Genome organization

All herpesviruses contain linear, double-stranded DNA genome surrounded by a 100-nm diameter icosahedral protein capsid core consisting of 162 capsomers (Lee *et al*., 2000). 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).

Meq is the most consistently expressed MDV gene expressed in transformed cells (Jones *et al.*, 1992). Meq is a 339 a. a. monocistronic protein, which expressed only by MDV-1 strains in transformed cells and tumor cells lines (Jones *et al.*, 1992). Mild and virulent MDVs (m/vMDVs) from the 1960s and 1970s encode a larger form of Meq, which is 398 a. a. and these contain reiterations of a C-terminal, proline-rich repeat (PPR) domain (Shamblin *et al.*, 2004). Meq is comprised of a basic DNA binding domain at its N-terminus followed by a leucine zipper dimerization motif (bZIP). Meq has a long C-terminal transactivation domain composed of proline-rich duplications followed by a 39 a.a. activation domain (Qian *et al.*, 1995).

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 (UL) and Short (US) regions are flanked by inverted and terminal repeat long and short regions (IRL, IRS, TRL, and TRS). (Source: Tulman *et al*., 2000).

2.6. Pathogenesis

The Cornell Model describes the pathogenesis of MD with four phases: early cytolytic, latency, secondary cytolytic, and transformation (Calnek, 1986).

2.6.1 Early cytolytic phase

MDV is taken into chickens through inhalation of infectious dander, and enters the circulation via phagocytosis by the lung epithelium, which transfer the virus to recruited macrophages and B-cells (Baaten *et al.,* 2009). The virus genome is detectable in primary and secondary lymphoid tissues, bursa of Fabricius, thymus, spleen and cecal tonsils, as early as 2-7 dpi, accompanied by expression of MDV early protein pp38 and lymphocytolysis with necrosis and infiltration of inflammatory cells (Baigent *et al.*, 1996). Macrophages have been shown to support some level of lytic infection and increased infection of macrophages has been seen with higher virulent MDVs (e.g. C12/130) (Barrow *et al.,* 2003). The main target cells of this early lytic phase are B cells, which are substantially eliminated in bursa of Fabricius and circulation (Shek *et al.,* 1983). However, B cells in the spleen are not essential for MDV replication, as surgical removal of spleens either had no effect on the appearance of latently-infected blood lymphocytes, nor did it abolish lymphoma development (Schat, 1981).

During this lytic phase of infection, the activation of T cells, particularly CD4+ cells, is observed. During this phase, no infectious cell-free virus is produced, and it is still unknown how the virus spreads between cells. However, Wu et al. shows that gH/gL complex is essential for spreading between uninfected cells (Wu *et al.*, 2001.), and others have demonstrated that gB

(Schumacher *et al.,* 2000), gE/gI, gM/gN and VP22, are essential for MDV cell-to-cell spreading (Tischer *et al.,* 2002).

2.6.2 Latency

Following the early cytolytic phase of infection, MDV establishes latency in T-cells, primarily CD4+ T-cells (Shek *et al.,* 1983). The definition of herpesvirus latency is the persistence of the viral genome in infected cells without the production of infectious virus. By 6-7 dpi, the MDV lytic antigen expression subsides in lymphoid organs and the switch from early lytic phase to latency phase starts. The host immune response to lytic infection has been shown to play an essential role in switching from cytolysis to latency (Buscaglia *et al.,* 1988). The role of host factors in latency is supported by the findings that immunosuppression prior to infection with MDV leads to prolonged early lytic infection, and chemically-induced immunosuppression after latency leads to reactivation and cytolytic infection. Latency-maintaining factor (LMF) and host cytokines, as well as soluble mediators (nitric oxide, NO), are involved in maintaining latency (Buscaglia *et al.,* 1988). In addition, unlike the early lytic phase, the predominant infected cells in latency are CD4+ TCR αβ+ T cells, which can be detected as early as 3 dpi.

During latency, transcription of the viral genome is limited to latency-associated transcripts (LATs), a complex family of spliced RNAs localizing to nucleus, that is abundant in MDV-transformed cells, but reduced upon MDV reactivation (Cantello *et al.,* 1997). Meq also plays a role in maintaining latency by blocking apoptosis of CD4+ cells (Levy *et al.,* 2005), transactivating latent gene expression (Parcells *et al.,* 2003.), and suppressing the promoters of MDV lytic genes ICP4 and pp38/pp14 (Levy *et al.,* 2003).

2.6.3 Secondary cytolytic phase

The virus undergoes reactivation and another semi-productive infection and cytolysis cycle approximately 3 - 4 weeks post-infection, coincident with the immunosuppression associated with proliferation of latently-infected CD4+ T-cells (Calnek, 1986). Although it is still unknown what causes the virus switch from latency to secondary cytolytic phase, it has been confirmed that once the virus is reactivated from latency, the virus replicates within the feather follicle epithelium (FEE) and will be shed to the environment in the dander, where it can remain infectious essentially indefinitely (Calnek, 1970). Expression of glycoprotein D (gD) was believed to be associated with virus shedding, as it is only expressed in FFE in vivo (Niikura *et* *al.*, 1999). However interruption of the gD-encoding gene (US6) does not prevent horizontal transmission (Anderson, *et al.*, 1998). More recently, glycoprotein C (gC) and the protein kinase encoded by the UL13 gene was found to be essential for horizontal transmission of MDV (Jarosinski *et al.*, 2007). However, the mechanisms regulating MDV productive infection at this site are currently unknown.

2.6.4 Transformation

During the secondary cytolytic phase, the transformation phase of infection becomes apparent, in which latently-infected CD4+ T cells proliferate and give rise to lymphomas. The spleen is a primary site for lymphomas formation, however, it is not essential for this, because splenectomized birds still develop tumors (Schat, 1981). Lymphomas have been observed in multiple organs, including the spleen, kidney, liver, intestine, and skin. MDV-transformed cells are a mixed population of T-lymphocytes expressing CD30, Marek's EcoRI-Q-encoded protein (Meq), MHC-II, and Marek's-associated tumor surface antigens (MATSAs) (Lee *et al.*, 2008). CD30 is a host-encoded surface antigen, which is over-expressed in EBV-associated human lymphomas such as Hodgkin's lymphoma, in which it is called the Reed-Sternberg antigen (Burgess, *et al.*, 2004). The proposed function of CD30 in tumor formation is that it contributes to polarization of the host immune response to a TH2 or humoral response, an ineffectual response in anti-tumor immunity (Shack *et al.*, 2008). Thus, MDV-transformed T-cells appear to have a TH3 or T-regulatory (Treg) immunophenotype (Shack *et al.*, 2008).

The relationship between latently infected cells and transformed cells is unclear, but it is confirmed that the episomal and integrated copies of MDV genome co-exist (Delecluse *et al.*, 1993), with integrated forms being much more common. The site of integration is randomly distributed and transformation is associated with the integration sites near telomeres of the host's mini-chromosomes and larger chromosomes (Delecluse *et al.*, 1993). Integration of the MDV genome may inhibit the expression of cytolytic gene expression and/or enhance the latency and transformation associated gene transcription. In a recently-described model for MDV latency, the MDV genome was found to integrate in an REV-transformed T-cell line and remain hypomethylated, indicating that DNA methylation is not directly involved in the silencing of lytic-phase genes during latency (Arumugaswami *et al.*, 2009).

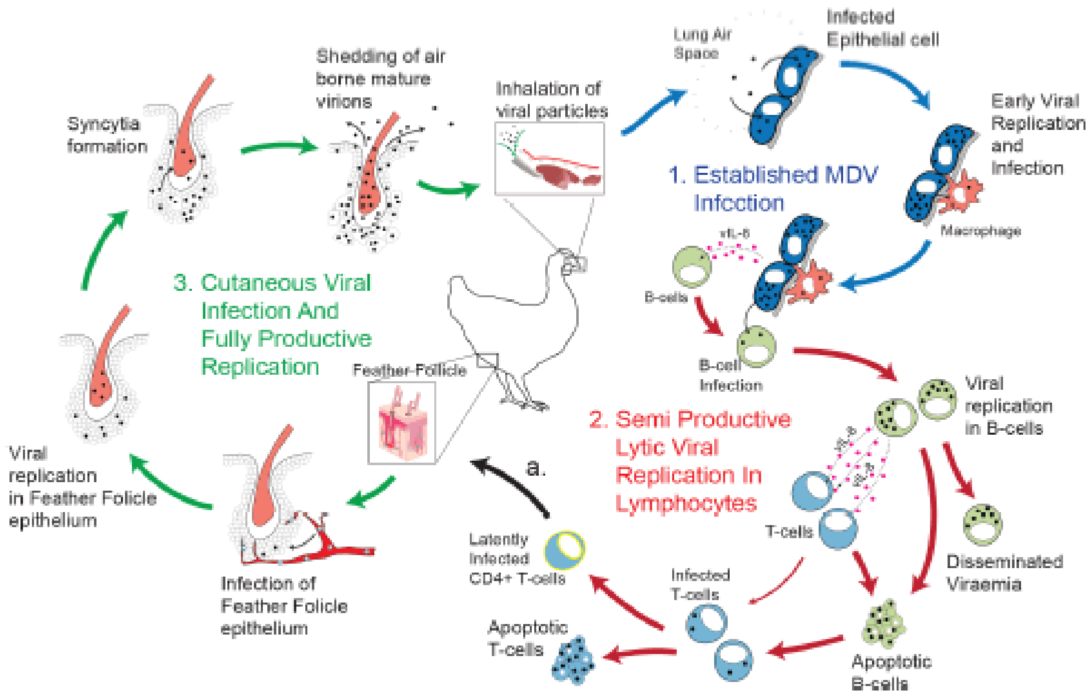

Figure 3: Pathogenesis of MD.

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

2.6.1. 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.

2.6.2. 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).

2.7. 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).

2.7.1. 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.

Laboratory confirmation 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 (AGPT), 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 tumors.

2.7.2. Recent advances in Marek's disease diagnosis

2.7.2.1. 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).

2.7.2.2. 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).

2.7.2.3. 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).

2.7.2.4. 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 is 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.

2.7.2.5. Loop mediated isothermal amplification technique

Loop mediated isothermal amplification technique 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 specificity, 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).

2.8. Distribution

2.8.1 Worldwide distribution of Marek's disease

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).

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 worldwide. 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).

The questionnaire survey which were used for the analysis of the incidence of MD in 55 countries revealed that only five countries, including Peru in Latin America, reported economic losses caused by MD since the 1990´s (Gimeno, 2004). 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. MD outbreaks were also reported in a few European countries including Russia with economic losses as well as in China. MD is only occasionally diagnosed in the Americas and the incidence is decreasing in most of South, Central and North America with the exceptions of Bolivia, Columbia, Peru, Uruguay and Venezuela. 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.

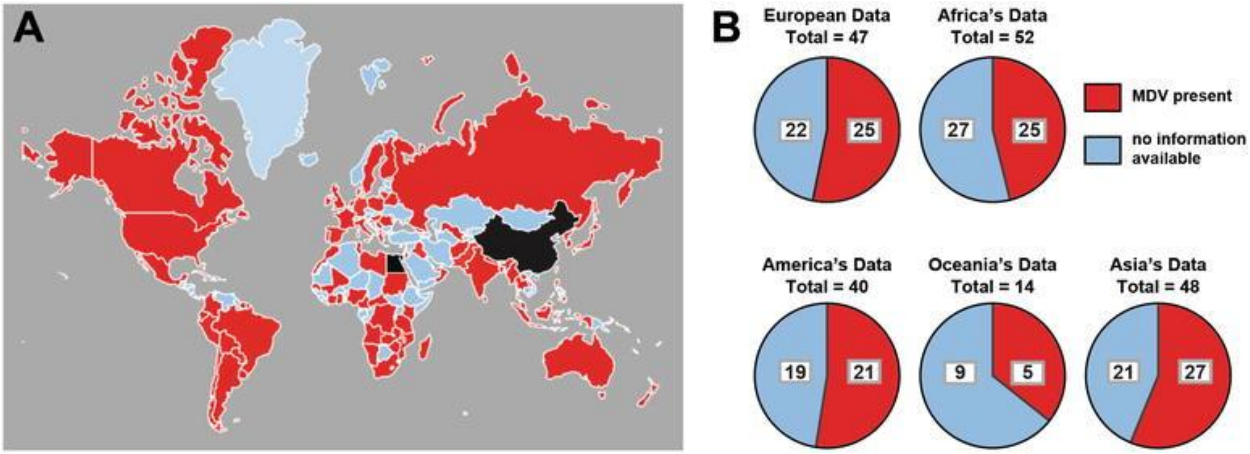

Figure 4. Worldwide map depicting distribution of MDV whereby evidence for presence has been submitted to the OIE (2014).

Distribution data was obtained from the World Organization for Animal Health (OIE) distributed through the World Animal Health Information Database (WAHID) interface and summarized above based on absence of information (blue) and presence of disease with reported cases (red) before and after 2009. **A** World map depicting countries positive for MDV was constructed using the imapbuilder software. Both China and Egypt (black) are endemic areas for MDV with outbreaks reported on a yearly basis. **B** Pie chart demonstrates the number of countries that have reported MDV cases to the OIE based on their geographical location (Figure 4).

2.8.2. 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, 340/503 and 333/503 respectively, indicating that MD is highly fatal (97.9% fatality) to the local breeds. The study was conducted at Debre 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 was widely distributed in the county and call for detail prevalence and epidemiological investigation. Isolation and molecular detection of MDV from outbreak samples was undertaken around central Ethiopia (Mirtneh, 2015) which revealed that from a total of 46/30 pooled outbreak samples subjected to conventional PCR test, 28 samples (93.3%) were positive for MD. Study conducted by Berhan (2014) on isolation and molecular characterization of Marek’s disease virus from clinically diseased chickens reared under different production system at central Ethiopia revealed that all Marek’s disease virus isolates were clustered under *Gallid Herpes virus* type 2.

2.9. Control and Prevention of Marek's Disease

2.9.1. Vaccination

Modified live vaccines are the cornerstone of current MD control programs. In the U.S., seven vaccine strains are currently licensed (FC126 (HVT), SB-1, 301B/1, CVI988 clone C, CVI988/C/R6, CVI988 (Rispens) and Md11/75). Additional strains are licensed in other countries. The vaccine strains may be used singly or in combinations that are of three general types or formulation in ascending order of efficacy: 1) HVT alone, 2) HVT plus a Serotype 2 strain, and 3) CVI988 (Rispens) with or without viruses of Serotypes 2 or 3. The CVI988 (Rispens) strain appears to be the most efficacious of the Serotype 1 strains and, at present, is widely used in layer and breeder chickens (Witter, 1997).

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 programs. These are usually administered to day-old chicks at hatching to provide protection against the natural challenge the chicks are exposed to early in life from the infected poultry house environment. With the introduction of in ovo immunization 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.

2.9.2. Biosecurity

Good biosecurity is very important to ensure that vaccinated chicks will develop immunity before they are subjected to a severe challenge of virus particularly for commercial flocks. 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. The use of vaccines should never be an excuse for poor management or lack of biosecurity measures (Murphy *et al.*, 1999, Venugopal *et al.*, 2001).

2.9.3. Selection for genetic resistance

Acquire families of birds and use sires that are resistant to MD, because there is strong level of heritability for genetic resistance against MD. It is one of the best ways to prevent Marek's Disease by developing a family of birds that is naturally resistant against the virus. 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

The present study was conducted from November, 2016 to May, 2017 on clinical samples which mainly consists of spleens and feather follicles which were collected from MD suspected chicken reared under semi-intensive and intensive poultry farms located at different regions of the country including Mekelle, Kombolcha, Addis Abeba, Bishoftu, and Adama within the project period. Bishoftu is located in Oromiya National Regional State about 45 KM south east of Addis Ababa and is at an altitude of 1850 meter above sea level. This area experiences a bimodal rainfall pattern with a short rainy season from March to May and a long rainy season from June to October. The area has an average annual rainfall of 800 mm and average maximum and minimum temperature of 28^{o}Cand 12.3^{o}C, respectively (CSA, 2015). It is found at 9^{o}N latitude and 40^{o}E longitude.

Addis Ababa is the capital and largest city of Ethiopia. The city has a complex mix of highland climate zones, with temperature differences of up to 10 °C (18 °F), depending on elevation and prevailing wind patterns. The city is located at 9°1′48″N 38°44′24″E with an average annual rainfall is about 1,165mm. From its lowest point, around at 2,326 meters above sea level in the southern periphery, the city rises to over 3,000 meters to the north.

Adama city is about 95kms south east of the Ethiopian capital, Addis Ababa. The city is located in Ethiopian rift valley, 39.1^{0} N and 8.31^{0} E at an elevation from 1590-1770 meters above sea level. Its annual rainfall ranges from 400-800 mm and annual mean temperature is 22^{0} C. The city has a total area of $9,616,399.5m^{2}$.

Kombolcha is located in the Debub Wollo Zone of the Amhara Region of the north-central Ethiopia and it has a latitude and longitude of 11°5′N 39°44′E with an elevation between 1842 and 1915 meters above sea level. It is found 375 km North of Addis Ababa. The highest annual rain fall is about 1313.6 mm and the lowest annual rain fall is 598.5 mm. The average annual temperature of the area is 20.1^{0}C.

Mekelle city is the administrative capital of Tigray National Regional State of Ethiopia. It is located at the northern part of the country at a distance of 870 km from the capital Addis Ababa.

It is found in 39° 28’ East and 13° 28́’ North at an average altitude of about 2084 meters above sea level, with an average mean temperature of 19°c and the annual rain fall varying between 50 to 250 mm.

The total chicken population in the country is estimated to be 56.87 million with native chicken representing 96.9%, hybrid chicken 0.54% and exotic breeds 2.56% (CSA, 2014). The population of poultry in regional perspective where the study areas are located is estimated to be 6.18 million, 18.03 million and 20.07 million in Tigray, Amhara and Oromiya regional states respectively. Poultry in Ethiopia, for the most part, is a backyard operation that uses a few scavenging birds that are of low productivity. The eggs and meat thus produced fall far short of meeting the growing local demand for eggs and poultry meat in the urban areas. The steady escalation of the prices for both eggs and meat is indicative of the growing local demand (CSA, 2014).

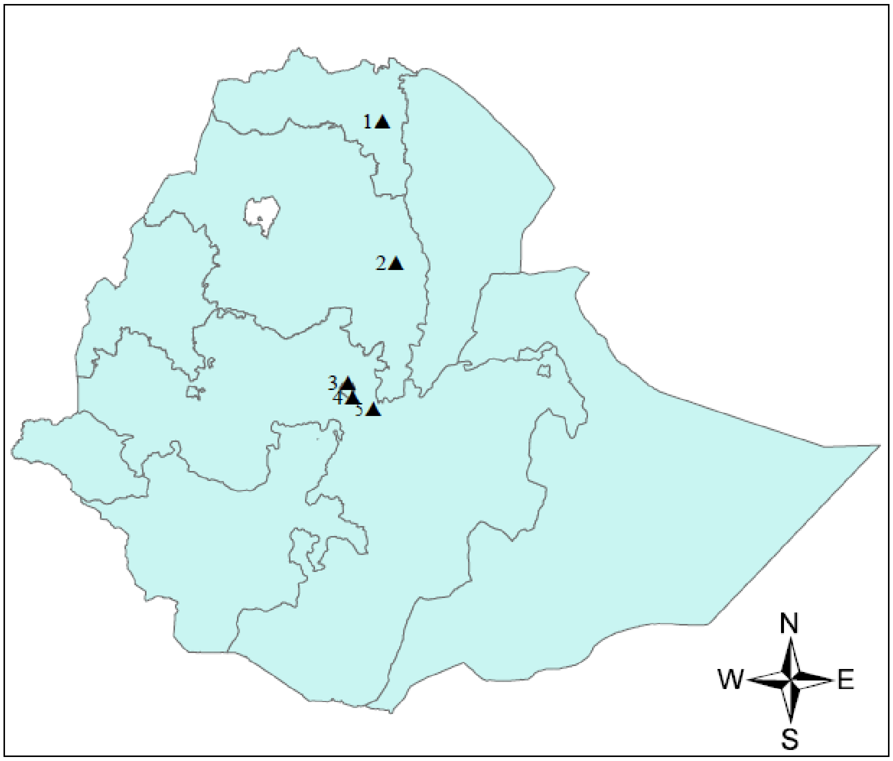

Figure 5. Map of Ethiopia showing the study areas where MD outbreak samples were collected from clinically diseased chickens. Where: 1= Mekelle, 2=Kombolcha, 3=Addis Ababa, 4=Bishoftu and 5= Adama.

3.2. Study Animals

The study was conducted in chickens that had experienced outbreaks of Marek’s disease. A total of 33 clinically sick chickens of all ages and breeds reared under back yard and commercialized cage production and management system were examined in the study.

3.3. Study Design

The study focused on cross sectional design mainly following a suspected outbreak of Marek’s disease. The result of this study was based on virus isolation and molecular and phylogenetic characterization of Marek’s disease virus.

3.4. Sampling method

Purposive sampling method was followed during the research period by which sampling was 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 for disease diagnosis and from field based on outbreak reports.

3.5. Sample Collection and Transportation

Sample collection was 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 and 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 at the post mortem facilities of the institute. Samples were placed in sterile and labeled 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).

3.6. Laboratory Investigation

3.6.1. 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, 3ml 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 will be centrifuged for 10 minutes at 1000 rpm. The supernatant was poured off and the cells will be re-suspended with Glasgow minimum essential medium (GMEM). The cell suspension will be transferred into a 25cm2 plastic tissue culture flask and kept at 37ºC incubator.

3.6.2. Virus isolation

3.6.2.1 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. Spleen samples that were collected from the same outbreak farm will be 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, $+4^{o}C$ 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. Those that had not showed CPE were blindly passage up to the third passage. Samples do not develop CPE until the third blind passage were considered as negative, whereas samples revealed characteristics CPE were considered as positive and kept at $-20^{o}C$ for further molecular analysis.

3.6.2.2. Virus isolation from feather

Feather follicles from individual affected chicken were collected and 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 with short (30 sec) interval to maintain the temperature. 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 have not showed CPE were blindly passage up to the third passage. Samples which were not develop any CPE until the third blind passage were considered as negative, whereas samples revealed characteristics CPE were considered as positive and kept at -20ºC for further analysis by molecular and sequencing techniques (Wozniakowski *et al*., 2011).

3.7. 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 were carried out using DNeasy® Blood and Tissue Kit (QIAGEN, Germany) following the manufacturer's instruction. Accordingly, 200µl tissue suspension was transferred into a labeled 1.5 ml microcentrifuge tube. 20 µl proteinase K and 200 µl Buffer AL were added for each tube and mixed by vortexing and incubated at 56ºC for 30 minutes (until completely lysed). 200µl 96% ethanol were added per tube and mixed thoroughly gently by vortexing. The mixture was transferred to a labeled 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 labeled 1.5ml eppendorf tube and

40 μl Buffer AE was added to the column and the nucleic acids bound to the silica membrane were eluted and the eluted DNA yield were used for PCR amplification.

3.8. 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 were performed targeting the amplification of the partial sequence of Meq gene of MD virus (600bp) using the MDV1OP forward primer and reverse primer as described by Handberg et al., 2001 (table 2). The primers were synthesized by VBC Biotech (Vienna, Austria) and purified by reverse phase high-performance liquid chromatography. The PCR were 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.

Table 2. Polymerase chain reaction (PCR) primers for Marek's disease virus (MDV) strain identification.

Target	Primer Sequence	Product Size (bp)
ICP4 gene	M1.1 F: 5'-GGATCGCCCACCACGATTACTACC-3'	318bp
	M1.8 R: 5'- ACTGCCTCACACAACCTCATCTCC-3'	
Meq gene	MDV1OP F: 5'GTCGACTTCGAGACGGAAAA-3'	600bp
	MDV1OP R: 5'CAGAAGAGGGAATGGGGAGT-3'	

Source: Handberg et al., 2001.

3.9. Agarose Gel Electrophoresis of Polymerase Chain Reaction Products

The polymerase chain reaction products were analyzed 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 pre-prepared gel and 100bp DNA molecular marker was also be added onto the first lane and run at 100 volt for about 60 minutes in electrophoresis apparatus. The DNA bands were visualized by gel documentation and the size of the PCR products were estimated by comparing with the band size of the molecular marker.

3.10. Sequencing and Phylogenetic Analysis

Polymerase chain reaction positive product were purified individually using Wizard® SV Gel and PCR product purification kit (Promega, Germany) following the manufacturer's instruction. The purified virus DNA concentrations were quantified using microvolume spectrophotometer (Nanodrop 2000c, USA). The concentrations of the quantified purified PCR products were adjusted following the requirements set by the sequencing company. The forward and reverse sequencing primers were added into separate labeled eppendorf tubes 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 were edited, blasted, aligned and phylogenetic tree was constructed including the reference strain sequences (n=14) retrieved from the Genbank (Table 3) using MEGA6 bioinformatics tool.

Table 3: Reference MD virus sequences retrieved from the Genbank

Number	Accession number
1	KC243262
2	KC243263
3	KC243264
4	KF895032
5	KJ949618
6	KP342383
7	KP342384
8	KP888821
9	KT246101
10	KT246102
11	KY651231
12	KY651232
13	KY651235
14	LC195188

Source: GenBank (NCBI).

3.11. Data Analysis

All generated Marek's disease virus Meq partial gene nucleotide raw sequence data were cleaned and contig were 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 (Tamura et al., 2013). A homologous region of 600bp nucleotide length corresponding to the partial sequence of Meq gene was used for phylogenetic tree construction using the different applications found in MEGA6 software (Tamura et al., 2013).

4. RESULTS

4.1. Field clinical examination

In this study a total of 33 chickens were clinically examined: 5 from Kombolcha, 3 from Mekelle, 12 from Bishoftu, 7 from Addis Ababa and 6 from Adama showing clinical signs of Marek's disease (Figure 5) were clinically examined and representative spleen and feather samples were collected (Table 4).

Table 4. Clinically diseased chickens examined for MD and Post mortem samples

Town	Type of organ		Vaccination history with HVT strain
	Spleen	Feather	
Bishoftu	6/12	30	Vaccinated
	6/12	30	Unvaccinated
Kombolcha	5	25	Vaccinated
Mekelle	3	15	Vaccinated
Adama	6	30	Vaccinated
Addis Ababa	7	35	Unvaccinated
Total	**33**	**165**	**198**

Clinical examinations were conducted on MD suspected chickens from Bishoftu, Kombolcha, Mekelle, Adama and Addis Ababa. The diseased birds showed clinical signs of paralysis (leg and wing), gray eye color, weight loss, labored breathing, depression and death (Figure 6). The clinical signs were mostly appeared in chickens with 4 to 16 weeks of age.

Figure 6: Clinically diseased chickens suspected of MDV infection

4.2 Post mortem Findings

Post mortem examinations were conducted on 10 chickens: 1 from Kombolcha, 1 from Mekelle, 3 from Bishoftu, 3 from Addis Ababa and 2 from Adama. Post mortem examination revealed that enlargement of the spleen as well as gross lesions in the visceral organs such as heart and liver were observed on 8 (80%) chickens from all sample areas and 2 (20%) of them which were 1 from Addis Ababa and 1 from Adama show no gross lesion. The gross lesions in the visceral organs had firm consistency, smooth surfaces, grey colored as well as nodular forms with varying degree in size.

4.3. Marek's Disease Virus Isolation

A total of 198 samples, 33 spleens and 165 feather follicles, were collected from 33 chickens of different outbreaks. Samples were collected from different poultry farms located in Bishoftu (12 spleen and 60 feathers), from Kombolcha (5 spleen and 25 feathers), from Mekelle (3 spleen and 15 feathers), from Adama (6 spleen and 30 feathers) as well as from Addis Ababa (7 spleen and 35 feather). Out of 7 pooled spleen samples, viruses were isolated from 5 (71.4%) preparations and also out of 10 pooled feather samples, viruses were isolated from 9 (90%). All the isolates that were grown and developed CPEs shows 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. Plaque were formed later by the cells that form foci and syncytia that detached from the wall of cell culture flask (Figure 7).

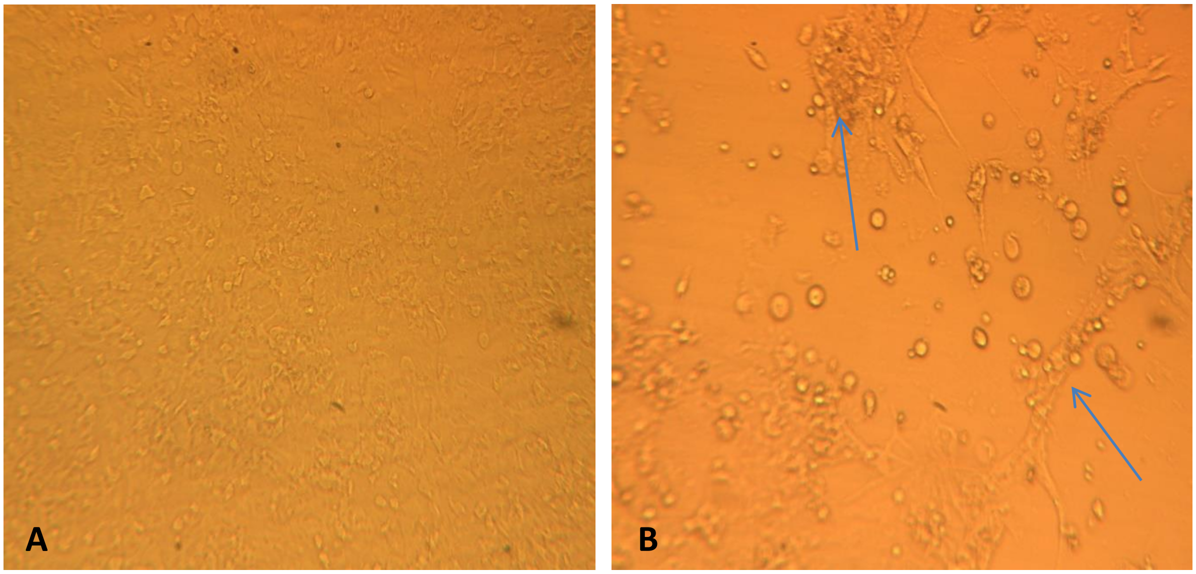

Figure 7. MD virus growth on chicken embryo fibroblast cells.

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

4.4. Detection of viral DNA by conventional Polymerase Chain Reaction

PCR amplification for ICP4 gene of MDV by the extracted virus DNA from field samples with the forward primer M1.1 and reverse primer M1.8 were performed to verify the specific presence of an approximately 318bp long DNA product of ICP4 gene. Positive samples in cell culture were pooled in their respective sample areas and prepared to run PCR. Seven pooled spleens as well as feather samples suspension were tested for MDV genome. Six of the pooled samples confirmed the presence of 318bp PCR products (Figure 8) and samples which were negative for virus isolation were also found negative in PCR (Table 5). Again 5 pooled samples in their respective sample areas were amplified for Meq gene using MDV1OP forward primer and reverse primer and result revealed that all are confirmed to be MDV positive (Figure 9).

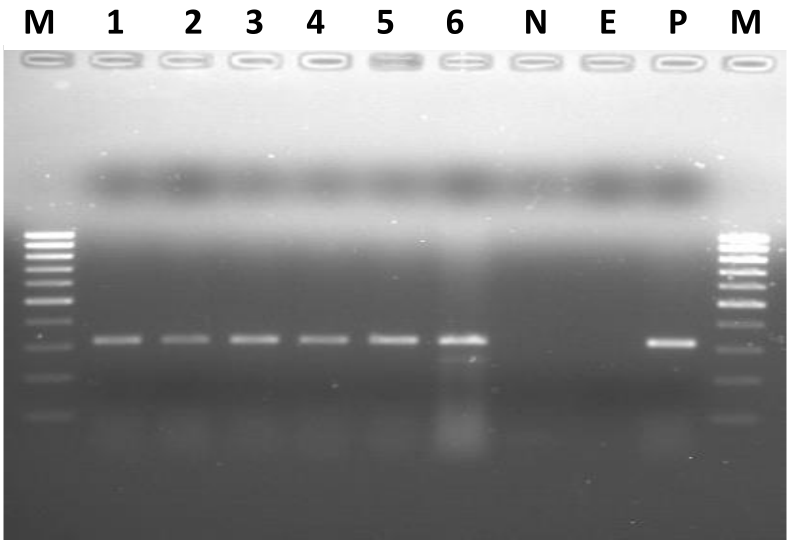

Figure. Agarose gel electrophoresis pattern of MDV partial ICP4 gene PCR products (approximately 318bp). Where, Lane M= 100bp DNA molecular marker (Fermentas); Lane 1- 6: Field samples; Lane N: Molecular grade water as negative control; Lane E: DNA extraction control; Lane P: Known MDV as positive control.

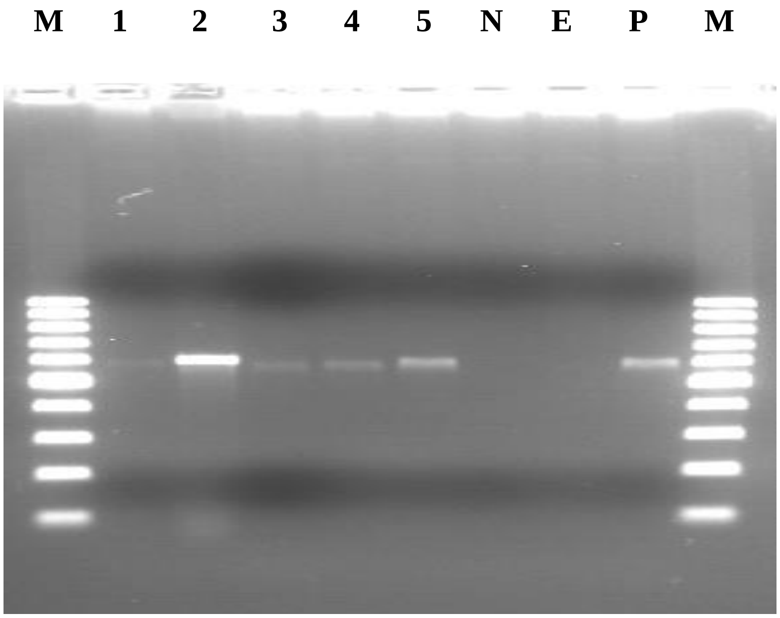

Figure. Agarose gel electrophoresis pattern of MDV partial Meq gene PCR products (approximately 600bp). Where, Lane M= 100bp DNA molecular marker (Fermentas); Lane 1- 5: Field samples; Lane N: Molecular grade water as negative control; Lane E: DNA extraction control; Lane P: Known MDV as positive control.

Table 5. Gene specific PCR results of outbreak samples tested for MDV

Sampling area	Pooled sampled tissue	Result	
		Positive	**Negative**
Bishoftu	2	1	1
Kombolcha	1	1	-
Mekelle	1	1	-
Adama	1	1	-
Addis Ababa	2	2	-
Total	**7**	**6**	**1**

4.5. Sequencing and Phylogenetic Tree Analysis

The purified PCR products from Addis Ababa (Akaki) and Bishoftu MD suspected outbreak isolates were sequenced and the sequence analysis showed that the two isolates have 100% sequence similarity and both cluster into Gallid Herpesvirus-2 serotype 1 (Figure 10). The currently sequenced two Ethiopian isolates of MDV/Akaki/14/2017/Meq gene and MDV/Bishoftu/3/2017/Meq gene were more closely related to the MDV isolates retrieved from the GenBank (KP342388, KF895032, KP342384, KT246101, KP888821, LC195188, KY651231, KY651232, KY651235 and KT246102).

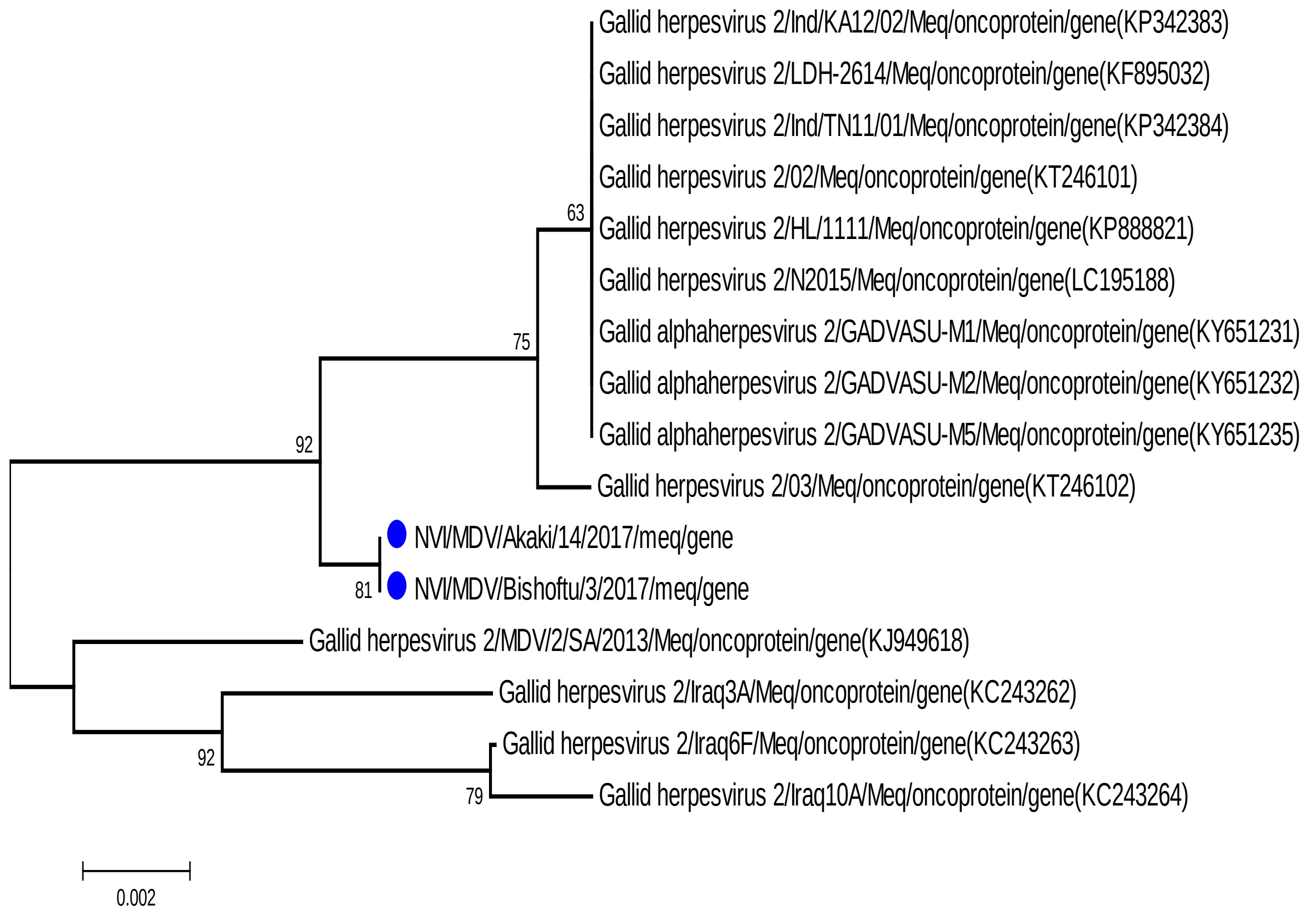

Figure. Phylogenetic analysis of the Meq oncoprotein coding sequence of MDV isolates.

The Neighbor-Joining method with the maximum composite likelihood nucleotide substitution model and the pairwise deletion option was computed using MEGA6. The genetic relationship between the MDV isolates of the current Ethiopian isolates with the previously characterized reference isolates retrieved from the GenBank was included in the analysis. The analysis involved 16 nucleotide Meq gene coding sequences. The percentage bootstrap scores above 50% (out of 1000 replicates) are shown next to the branches. The current sequenced isolates were indicated in color circle.

4.6. Nucleotide variability

Sequence alignment analysis revealed nucleotide variability and similarity among the analyzed samples and reference strains (Fig.11). The two sample isolates (Akaki and Bishoftu) were similar to each other and both are almost similar with few mismatches compared with reference sequences of Gallid herpesvirus 2 strains.

```
                                         10        20        30        40        50        60
                                 ....|....|....|....|....|....|....|....|....|....|....|....|
MDV/Akakai/14/2017/Meq           CGGAAAAAAAGGAAAAGTCACGACATCCCCAACAGCCCCTCCAAACACCCCTTCCCTGAC
MDV/Bishoftu/3/2017/Meq          ............................................................
GH-2/MDV/2/SA/2013/Meq(KJ949618) ............................................................
GH-2/MDV/Iraq6F/Meq(KCKC243263)  ............................................................
GH-2/MDV/Iraq10A/Meq(KCKC243264) ............................................................
GH-2/MDV/Iraq3A/Meq(KCKC243262)  ........................................................C...
GH-2/MDV/GADVASU-M5/Meq(KY651235)............................................................
GH-2/MDV/GADVASU-M2/Meq(KY651232)............................................................
GH-2/MDV/GADVASU-M1/Meq(KY651231)............................................................
GH-2/Meq(LC195188)               ............................................................
GH-2/HL/1111/Meq(KP888821)       ............................................................

                                         70        80        90       100       110       120
                                 ....|....|....|....|....|....|....|....|....|....|....|....|
MDV/Akakai/14/2017/Meq           GGCCTATCTGAGGAGGAGAAACAGAAGCTGGAAAGGAGGAGAAAAAGGAATCGTGACGCC
MDV/Bishoftu/3/2017/Meq          ............................................................
GH-2/MDV/2/SA/2013/Meq(KJ949618) ............................................................
GH-2/MDV/Iraq6F/Meq(KCKC243263)  ............................................................
GH-2/MDV/Iraq10A/Meq(KCKC243264) ............................................................
GH-2/MDV/Iraq3A/Meq(KCKC243262)  ............................................................
GH-2/MDV/GADVASU-M5/Meq(KY651235)............................................................
GH-2/MDV/GADVASU-M2/Meq(KY651232)............................................................
GH-2/MDV/GADVASU-M1/Meq(KY651231)............................................................
GH-2/Meq(LC195188)               ............................................................
GH-2/HL/1111/Meq(KP888821)       ............................................................
                                        130       140       150       160       170       180
                                 ....|....|....|....|....|....|....|....|....|....|....|....|
MDV/Akakai/14/2017/Meq           GCTCGGAGAAGACGCAGGGAGCAGACGTACTATGTAGACAAACTCCATGAAACATGTGAA
MDV/Bishoftu/3/2017/Meq          ............................................................
GH-2/MDV/2/SA/2013/Meq(KJ949618) ...................................................G........
GH-2/MDV/Iraq6F/Meq(KCKC243263)  T..........................G.......................G........
GH-2/MDV/Iraq10A/Meq(KCKC243264) T..........................G.......................G........
GH-2/MDV/Iraq3A/Meq(KCKC243262)  ...................................................G........
GH-2/MDV/GADVASU-M5/Meq(KY651235)............................................................
GH-2/MDV/GADVASU-M2/Meq(KY651232)............................................................
GH-2/MDV/GADVASU-M1/Meq(KY651231)............................................................
GH-2/Meq(LC195188)               ............................................................
GH-2/HL/1111/Meq(KP888821)       ............................................................

                                        190       200       210       220       230       240
                                 ....|....|....|....|....|....|....|....|....|....|....|....|
MDV/Akakai/14/2017/Meq           GAGCTGCGGAGGGCCAATGAACACCTACGTAAGGAAATTCGAGATCTAAGGACTGAGTGC
MDV/Bishoftu/3/2017/Meq          ............................................................
GH-2/MDV/2/SA/2013/Meq(KJ949618) .......A.................................................A..
GH-2/MDV/Iraq6F/Meq(KCKC243263)  .......A....................................................
GH-2/MDV/Iraq10A/Meq(KCKC243264) .......A....................................................
GH-2/MDV/Iraq3A/Meq(KCKC243262)  .......A....................................................
GH-2/MDV/GADVASU-M5/Meq(KY651235)............................................................
GH-2/MDV/GADVASU-M2/Meq(KY651232)............................................................
GH-2/MDV/GADVASU-M1/Meq(KY651231)............................................................
GH-2/Meq(LC195188)               ............................................................
GH-2/HL/1111/Meq(KP888821)       ............................................................

                                        250       260       270       280       290       300
                                 ....|....|....|....|....|....|....|....|....|....|....|....|
MDV/Akakai/14/2017/Meq           ACGTCCCTGCGTGTACAGTTGGCTTGTCATGAGCCAGTTTGCCCTATGGCGGTACCCCTA
MDV/Bishoftu/3/2017/Meq          ............................................................
GH-2/MDV/2/SA/2013/Meq(KJ949618) ............................................................
GH-2/MDV/Iraq6F/Meq(KCKC243263)  ..............G.............................................
GH-2/MDV/Iraq10A/Meq(KCKC243264) ..............G.............................................
GH-2/MDV/Iraq3A/Meq(KCKC243262)  ........................C...................................
GH-2/MDV/GADVASU-M5/Meq(KY651235)............................................................
GH-2/MDV/GADVASU-M2/Meq(KY651232)............................................................
GH-2/MDV/GADVASU-M1/Meq(KY651231)............................................................
GH-2/Meq(LC195188)               ............................................................
GH-2/HL/1111/Meq(KP888821)       ............................................................
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                                        310       320       330       340       350       360
                                 ....|....|....|....|....|....|....|....|....|....|....|....|
MDV/Akakai/14/2017/Meq           ACGGTGACCCTTGGACTGCTTACCGCCCCGCACGATCCCGTTCCTGAACCTCCCATTTGC
MDV/Bishoftu/3/2017/Meq          ............................................................
GH-2/MDV/2/SA/2013/Meq(KJ949618) ........................A......C............................
GH-2/MDV/Iraq6F/Meq(KCKC243263)  ........................A...................................
GH-2/MDV/Iraq10A/Meq(KCKC243264) ........................A...................................
GH-2/MDV/Iraq3A/Meq(KCKC243262)  ........................A...................................
GH-2/MDV/GADVASU-M5/Meq(KY651235)............................................................
GH-2/MDV/GADVASU-M2/Meq(KY651232)............................................................
GH-2/MDV/GADVASU-M1/Meq(KY651231)............................................................
GH-2/Meq(LC195188)               ............................................................
GH-2/HL/1111/Meq(KP888821)       ............................................................

                                        370       380       390       400       410       420
                                 ....|....|....|....|....|....|....|....|....|....|....|....|
MDV/Akakai/14/2017/Meq           ACTCCTCCACCTCCCTCACCGGATGAACCTAACGCTCCACATTGCTCCGGTTCCCAACCT
MDV/Bishoftu/3/2017/Meq          ............................................................
GH-2/MDV/2/SA/2013/Meq(KJ949618) ............................................................
GH-2/MDV/Iraq6F/Meq(KCKC243263)  ............................................................
GH-2/MDV/Iraq10A/Meq(KCKC243264) ...................................................C........
GH-2/MDV/Iraq3A/Meq(KCKC243262)  ...................................................C........
GH-2/MDV/GADVASU-M5/Meq(KY651235)............................................................
GH-2/MDV/GADVASU-M2/Meq(KY651232)............................................................
GH-2/MDV/GADVASU-M1/Meq(KY651231)............................................................
GH-2/Meq(LC195188)               ............................................................
GH-2/HL/1111/Meq(KP888821)       ............................................................

                                        430       440       450       460       470       480
                                 ....|....|....|....|....|....|....|....|....|....|....|....|
MDV/Akakai/14/2017/Meq           CCTATCTGTACCCCCGCTCCTCCCGATGCGGAGGAGCTTTGCGCCCAGCTCTGCTCGACC
MDV/Bishoftu/3/2017/Meq          ............................................................
GH-2/MDV/2/SA/2013/Meq(KJ949618) ...............C............................................
GH-2/MDV/Iraq6F/Meq(KCKC243263)  ............................................................
GH-2/MDV/Iraq10A/Meq(KCKC243264) ............................................................
GH-2/MDV/Iraq3A/Meq(KCKC243262)  ............................................................
GH-2/MDV/GADVASU-M5/Meq(KY651235)...............C...........A.......A........................
GH-2/MDV/GADVASU-M2/Meq(KY651232)...............C...........A.......A........................
GH-2/MDV/GADVASU-M1/Meq(KY651231)...............C...........A.......A........................
GH-2/Meq(LC195188)               ...............C...........A.......A........................
GH-2/HL/1111/Meq(KP888821)       ...............C...........A.......A........................

                                        490       500
                                 ....|....|....|....|....|..
MDV/Akakai/14/2017/Meq           CCACCACCTCCCATCTCTACTCCCCAT
MDV/Bishoftu/3/2017/Meq          ...........................
GH-2/MDV/2/SA/2013/Meq(KJ949618) ................G..........
GH-2/MDV/Iraq6F/Meq(KCKC243263)  ................G..........
GH-2/MDV/Iraq10A/Meq(KCKC243264) ................G..........
GH-2/MDV/Iraq3A/Meq(KCKC243262)  ................G..........
GH-2/MDV/GADVASU-M5/Meq(KY651235)...........................
GH-2/MDV/GADVASU-M2/Meq(KY651232)...........................
GH-2/MDV/GADVASU-M1/Meq(KY651231)...........................
GH-2/Meq(LC195188)               ...........................
GH-2/HL/1111/Meq(KP888821)       ...........................
```

Figure 8. Plot identity for the Meq gene nucleotide sequences of Marek's Disease virus of the current Ethiopian isolates (n=2) from chickens in comparison with the reference strains (n=9) retrieved from the GenBank. Identical nucleotides are indicated as dot. Random nucleotide variations observed at different positions.

5. DISCUSSION

Marek’s disease is one of the most economically devastating infectious diseases of poultry commonly characterized by oncogenic transformation of T cells that infiltrates lymphoid tissues, peripheral nerves and visceral organs, resulting in complex pathogenesis that usually leads to the death of the affected birds. The present study demonstrated the isolation and molecular characterization of Marek’s disease virus from clinically diseased Ethiopian chickens reared under different production system. Confirmatory diagnosis of the virus was reached by using the recommended diagnostic techniques (OIE, 2010) of CEF cell culture, polymerase chain reaction and sequencing of isolates.

Clinical manifestations observed during the study period were numerous and varied, including leg and wing paralysis, gray eye color, weight loss, labored breathing, depression and high mortality. The clinical signs were mostly appeared in 4 to 12 weeks old chickens.

Gross lesions in the visceral organs such as heart and liver which are indicative of lymphoid tumors was observed in 8 (80%) chickens out of 10 chickens that were autopsied. The gross lesions in the visceral organs had firm consistency, smooth surfaces, grey colored as well as nodular forms with varying degree in size. These post mortem findings are in conformity with Fenner et al. (2011) and OIE (2010).

Marek's disease virus isolation on primary chicken embryo fibroblast were demonstrated on 198 different outbreaks samples, 33 spleens and 165 feather follicles collected from Kombolcha, Mekelle, Addis Ababa, Bishoftu and Adama. 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 and this 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 presence of MD and/or MDV has been reported 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). Isolation and molecular detection of MDV from outbreak samples was undertaken around central Ethiopia (Mirtneh, 2015) which revealed that from a total of 46/30 pooled outbreak samples subjected to conventional PCR test, 28 samples

(93.3%) were positive for MD. Study conducted by Berhan (2014) on isolation and molecular characterization of Marek’s disease virus from clinically diseased chickens reared under different production system at central Ethiopia revealed that all Marek’s disease virus isolates were clustered under *Gallid Herpes virus* type 2. However, all reports and research activities on the distribution of MD in the country were conducted in the central part of Ethiopia which encompasses most of the commercial poultry farms in the country that contributed only 2.56% (CSA, 2014) of the total chicken population. The present study endeavor to show the other side of the country where MD becomes the most serious constraint that hinders the poultry industry.

There are a lot of reports and MD suspected samples coming to National Veterinary Institute (NVI) on 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 despite vaccination with imported HVT F126 vaccine. This is the driving force for the present study to be conducted in order to confirm the circulating Marek's disease virus strains in the chicken population using the recommended diagnostic laboratory techniques among different regions of Ethiopia.

The present study also confirmed and demonstrated the clinical cases of MD and managed to isolate MDV on chicken with the history of vaccination against MD using HVT F126 vaccine. This is in agreement with recently published research activity suggesting that MD vaccination failure is probably due to the existence of variant MDV strains with known virulence and unexpected vaccine resistance by Guo-rong *et al.* (2017). Even though there have been so many factors that hinders the efficacy of the vaccine further investigation is actually call for developing MD vaccines using local isolates.

The viruses isolated from outbreak samples on CEF cell were confirmed to be MDV by using virus specific genotyping methods targeting to amplify an 318bp fragment of ICP4 gene of MDV using conventional polymerase chain reaction on agarose gel electrophoresis. 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. PCR analysis was performed using the forward primer M1.1 and reverse primer M1.8, which were designed as per the sequence published by Handberg et al. (2001) targeting a nucleotide sequence in ICP4 gene (unique for MDV-1).This PCR finding is in agreement with the previous report of Kalyani (2010), Mirtnehe

(2015) and Berhan (2014). In the fact that the same PCR product band size obtained using the amplification primers M 1.1 and M 1.8. Primer set M1.1/M1.8 amplified a 318 bp product are similar with the expected 247 bp product (Kalyani et al., 2010). PCR analysis was also performed using the forward primer MDV1OP 5'GTCGACTTCGAGACGGAAAA-3'and reverse primer MDV1OP 5'CAGAAGAGGGAATGGGGAGT-3', which strengthens the presence of Meq gene.

The present laboratory based research findings will contribute more information to the scientific communities on the existence of the Marek's disease in Northern (Mekelle), North-Central (Kombolcha) and Central (Addis Ababa, Bishoftu, Adama) regions of Ethiopia.

As 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 some of the study areas (Kombolcha and Mekelle) and the second in the central part of Ethiopia and confirmed the circulation of Marek's disease virus in chickens to the study areas.

Further study on the outbreak investigation of chicken MD in the other parts of the country as well as full genome sequencing of the present isolates will enrich the information about the circulating virus type which is important to reach in a decision on the selection of safe and protective vaccine strain since there have been high demand of MD vaccine in the country and about the nature of the virus such as serotype and pathotype respectively.

6. CONCLUSION AND RECOMMENDATIONS

In conclusion, the present study revealed that Marek’s disease virus were circulating in chicken flocks of Northern (Mekelle), North-Central (Kombolcha) and Central (Addis Ababa, Bishoftu, Adama) regions of Ethiopia and clustered under Gallid Herpes virus type 2. In understanding the molecular epidemiology of MD viruses in the poultry farms the present study is highly important for the design of preventive measures as well as for the development and production of effective vaccine for the control of the spread of the virus. Thus extra attention should be given by the Ministry of livestock and fisheries as well as by the poultry farm owners on the prevention and control of Marek’s disease which becomes a serious health problem of the poultry industry in Ethiopia.

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

- Further study on the isolation and molecular characterization of chicken Marek’s disease virus in all regions of the country as well as full genome sequencing of the present isolates is required to reach in a decision on the selection of safe and protective vaccine strain is important.
- Study on the relationship between poultry breeds (local and exotic) with virulence of Marek’s disease virus should be conducted.
- Investigations on field effectiveness of the imported MD vaccines need to be conducted.
- Commercial poultry farms should implement regular vaccination program, thereby reducing the impacts of MD.

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8. ANNEXES

Annex 1: Chicken Embryo Fibroblast Cell Preparation Protocol

1. Remove the embryos from 10-11 day-old eggs after having swabbed the tops of the shells with 70% alcohol.

2. Eviscerate the embryos, first cut off the head and limbs, and place in PBS at 37^0C.

3. Cut the remains of the embryos into small pieces in a Petri dish with scissors.

4. Transfer the tissue to a 150ml flask and add 20ml of PBS at 37^0C.

5. Wash the tissue until the PBS become clear. Pour off the PBS and discard.

6. Add 20ml of Trypsin solution at 37^0c, swirl and allow the tissue to settle. Pour off the supernatant and discard.

7. Add 20ml of Trypsin solution and place the flask on a magnetic stirrer in a bench incubator at 37^0C. Stir for 15 min at a rate sufficient to give through mixing.

8. Decant the supernatant cell suspension into a universal bottle containing needed amount foetal calf serum and mix.

9. Repeat the procedures in steps 7 & 8.

10. Filter the cell suspension through double layer of sterile muslin.

11. Centrifuge the cell suspension at 600 rpm for 5 min.

12. Using a 10ml pipette, re-suspend the cell pellets in 8 ml complete GMEM and pour into a 10ml graduated centrifuge tube.

13. Centrifuge the cell suspension at 900 rpm for 5 min.

14. Using a 10ml pipette, dilute the cells 1: 300 in complete GMEM (8 eggs should give approx. 1 ml of packed cells).

15. Dispense the cell suspension into the required vessels and incubate at 37^{0}C. (7.5ml for 25cm2, 25ml for 75cm2, and 50ml for 162cm2 flasks.

16. Confluence should be obtained within 24 hours.

Annex 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.

Annex 3: DNA extraction

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

1. Cut tissue (≤ 10mg spleen or ≤ 25mg other tissue) into small pieces, and place in a 1.5 ml microcentrifuge tube. Add 180µl Buffer ATL. Add 20µl proteinase K, mix by vortexing, and incubate at 56^{o}C until completely lysed. Vortex occasionally during incubation. Vortex 15s directly before proceeding to step 2.

2. Add 200 µl Buffer AL. Mix thoroughly by vortexing. Incubate samples at 56^{o}C for 10 min.

3. Add 200 µl ethanol (96-100%). Mix thoroughly by vortexing.

4. Pipet the mixture into a DNeasy Mini spin column placed in a 2 ml collection tube. Centrifuge at ≥ 6000 x g (8000 rpm) for 1 min. Discard the flow-through and collection tube.

5. Place the spin column in a new 2 ml collection tube. Add 500 µl Buffer AW1. Centrifuge for 1 min at ≥6000 x g. Discard the flow-through and collection tube.

6. Place the spin column in a new 2 ml collection tube. Add 500 µl Buffer AW2, and centrifuge for 3 min at 20,000 x g (14,000 rpm). Discard the flow-through and collection tube.

7. Transfer the spin column to a new 1.5 ml or 2 ml microcentrifuge tube.

8. 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^{o}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 45^{0}C 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.1µl loading buffer 5µl 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.

Annex 4. Phosphate buffered saline (PBS) without calcium or magnesium

Sodium Phosphate Dibasic (Na2HPO4)	1.60 gms
Potassium Phosphate (KH2PO4)	0.51 gms
Sodium Chloride (NaCl)	7.30 gms
Double distilled water	1 liter

Sterilized by Autoclave

Annex 5. Trypsin (0.25%) solution preparation

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 H20 q.s.	1 liter

Adjust pH to 7.4 with $NaHCO_3$ solution

Annex 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

Annex 7. Gel slice and PCR product preparation

A) Dissolving the gel slice

1. Following electrophoresis excise DNA band from gel and place gel slice in a 1.5ml microcentrifuge tube.
2. Add 10μl membrane binding solution per 10mg of gel slice. Vortex and incubate at 50-65^{0}C until gel slice is completely dissolved.

B) Processing PCR amplifications

Add an equal volume of membrane binding solution to the PCR amplification

Binding of DNA

1. Insert SV Mini column into collection tube
2. Transfer dissolved gel mixture or prepared PCR product to the minicolumn assembly incubate at room temperature for 1 minute
3. Centrifuge at 16,000 x g for 1 minute. Discard flowthrough and reinsert minicolumn into collection tube

Washing

4. Add 700 μl membrane wash solution (ethanol added) centrifuge at 16,000 x g for 1 minute discard flow through and reinsert minicolumn into collection tube
5. Repeat step 4 with 500 μl membrane wash solution centrifuge at 16,000 x g for 5 minutes
6. Empty the collection tube and re-centrifuge the column assembly for 1 minute with the microcentrifuge lid open (or off) to allow evaporation of any residual ethanol.

Elution

7. Carefully transfer minicolumn to a clean 1.5ml microcentrifuge tube
8. Add 50 μl of nuclease free water to the minicolumn incubate at room temperature for l minute centrifuge at 16,000 x g for 1 minute
9. Discard minicolumn and store DNA at 4^{0}C or at -20^{0}C.