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ISOLATION AND MOLECULAR CHARACTERIZATION OF MAREK'S DISEASE VIRUS IN CENTRAL ETHIOPIA AND EVALUATION OF ITS VACCINE TRIAL

MSc. THESIS

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Department of Microbiology, Immunology and Veterinary Public Health

MSc program in Veterinary Microbiology

JUNE, 2015

BISHOFTU, ETHIOPIA

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

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As members of the Examining Board of the final MSc open defence, we certify that we have read and evaluated the Thesis prepared by: Mirtneh Akalu Entitled "Isolation and Molecular Characterization of Marek's Disease Virus in Central Ethiopia and Evaluation of Its Vaccine Trial" 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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ABBERVIATIONS

CEF	Chicken Embryo Fibroblast
CEK	Chicken Embryo kidney
CFM	Cell freezing medium
CPE	Cytopathic effect
DNA	Deoxyribonucleic Acid
GaHV-2	*Gallid herpesvirus* 2
GaHV-3	*Gallid herpesvirus* 3
HVT	Herpes Virus of Turkey
MD	Marek's disease
MDV	Marek's disease virus
MeHV-1	*Meleagrid herpesvirus* 1
NVI	National Veterinary Institute
OIE	Office International des Epizooties
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFU	Plaque Forming Units
TCID50%	Tissue Culture Infectivity dose 50
TEM	Transmission Electron Microscopy
VP	Viral protein

ABSTRACT

The present study was conducted to isolate Marek's disease virus from outbreak samples and to evaluate HVT FC 126 vaccine strain against local isolates. The outbreak investigation was performed in six purposively selected sites (Addis Ababa, Akaki, Dukem, Bishoftu, Mojo and Adama) on the basis of outbreak report. Complete random design was employed for pathogenicity and efficacy trial on Bovans brown chickens. A characteristics clinical signs of MD was observed on sick chickens. White foci of tumour on liver, heart and lung and enlargement of spleen were the post mortem findings of euthanized sick chickens. Based on vaccination history morbidity in non-vaccinated flocks ranges from 22.2% to 51% and mortality as high as 29%. In vaccinated flocks morbidity ranges from 0.7% to 3.3% and mortality as high as 0.4%. From 46/30 pooled samples collected 93.3% were confirmed to be MDV through virus specific genotyping methods, targeting the ICP4 gene of MD virus (318bp) using conventional PCR. 130 chickens were assigned in seven experimental groups to evaluate the pathgenicity of the six new cell culture-grown local isolates. Challenge was performed in two alternative doses (500 and 1000 pfu) by inoculating intraperitoneally at 8 day of age. Death was observed from 30% - 50% and post mortem lesion 50%-70% at 1000 pfu. No death and post mortem lesion was observed from 60%-80% at 500 pfu. Thus, all six isolates at 1000 pfu dose fulfilled the criterion to be standard challenge strain. The evaluation of the efficacy of trial vaccine formulated from HVT FC 126 was performed on 70 chickens. Chickens immunized at day old through subcutaneous route with 2000 pfu were challenged in respective of the six local isolates at 7 day of post vaccination. None of the chickens were dead while only Addis Ababa, Dukem and Adama isolates caused tumour lesions in one chicken from their group. The actual MD incidence was rated from 0-10% in challenged groups. The protective index reaches to 95%. Thus the trial vaccine provided efficient protection against MD. However, the vaccine effectiveness shall be evaluated in the context of the existing poultry production system.

Key words: Marek’s disease, chicken, pathogenicity, efficacy, vaccine, CPE, PCR, Ethiopia

1. INTRODUCTION

The poultry sector continues to grow and industrialize in many parts of the world. An increasing population, greater purchasing power and urbanization have been strong drivers of the growth. Advances in breeding and health care have given rise to birds that meet specialized purposes and are increasingly productive, but that needs professional management. The development and transfer of feed, slaughter and processing technologies have increased safety and efficiency, which favors large-scale units rather than small-scale producers. These developments have led the poultry industry to scale up rapidly (FAO, 2014).

In Ethiopia, Poultry production can be classified into three categories namely: traditional, small-scale and large-scale commercial farms. The former is based on indigenous chicken types whereas the later two production systems are characterized by more intensively managed exotic chicken lines (Alemu and Tadelle, 1997) and the major poultry products come from backyards chickens. But in recent times, more commercialized poultry farms are flourishing having considerable contribution to the supply of poultry products, especially to urban areas. There are also attempts to upgrade the productivity of local chickens through distribution of exotic and cross breeds to the rural areas. These endeavors, however, are hampered from providing the expected benefits due to various constraints, among which viral diseases are of greater concern (Hailu, 2012).

Research and case reports coming from various regions of the country indicated that viral diseases are posing a growing threat to the young poultry industry flourishing in the country. In addition, the intensification and dissemination of susceptible exotic breeds to villages has been exacerbating the prevalence of poultry viral diseases (Zeleke *et al*., 2005a). Newcastle disease, Infectious bursal disease and Marek's disease (MD) are among the serious threats to poultry production (Tadelle, 1996; Zeleke *et al*., 2005a). However, the epidemiology and the total economic damage caused by these diseases are not fully known. Frequent outbreaks and occurrence of new strains for these viral diseases became a challenge to the poultry industry in Ethiopia (Hailu, 2012).

Among these different viral diseases Marek's disease is one of the potential threats for the poultry production in the country (Berhan, 2014). Marek's disease is a devastating disorder affecting chickens worldwide. It is caused by Marek's disease virus (MDV), an Alphaherpesvirus, and is characterized by T cell lymphomas, polyneuritis, immunosuppression and, rarely atherosclerosis (Calnek, 2001).

In chickens, MD occurs at 3–4 weeks of age or older and is most common between 12 and 30 weeks of age. Clinical signs observed are paralysis of the legs and wings, with enlargement of peripheral nerves, but nerve involvement is sometimes not seen, especially in adult birds (Fenner *et al*., 2011). MDV strains of higher virulence may also cause increased mortality in young birds of 1–2 weeks of age, especially if they lack maternal antibodies. Depending on the strain of MDV, lymphomatosis can occur, especially in the ovary, liver, spleen, kidneys, lungs, heart, proventriculus and skin (OIE, 2010).

Marek's disease was first diagnosed in Ethiopia in 1983 and an incidence rate of 0.3% in industrial poultry farms was reported for the years 1983 – 1986 (Alamargot, 1987). On the other hand 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.

According to Duguma *et al.* (2005) the magnitudes of morbidity and mortality on indigenous chickens in Ethiopia were nearly equal, indicating that MD is highly fatal to the local breeds. A study conducted by Berhan (2014) on isolation and molecular characterization of pathological samples collected from different geographical areas of the country revealed that the circulating MD virus in chickens of central Ethiopia was clustered under *Gallid Herpes virus* type 2,MDV serotype 1, which is known as an oncogenic strain (Shambhu *et al*., 2012). All pathotypes of Serotype 1 cause disease (Witter, 1991).

Following the introduction of HVT vaccine about 1971, losses from MD in broiler and layer chickens were dramatically reduced (Witter, 1997). MD vaccination has significantly

increased the survival rate of the local breeds, this indicates the 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 (Duguma *et al.,* 2006).

Despite the growing importance of MD vaccine in Ethiopia, it is being availed in by importation (Berhan, 2014). These vaccines are produced from MD strains isolated from different production systems and because of this they may not be as effective as they are supposed to be (Churchill *et al*., 1969). Vaccination breaks might occur due to different factors such as improper handling of vaccine, lack of sterilizing immunity, poor flock management, influence of other pathogens which can cause immunosuppressive effects in the host, presence of stress (Adair 2000; Jeurissen *et al*., 1992).

Generally, vaccines acquired from other countries via foreign currency and with higher transportation cost makes the vaccine to be more costly (Berhan, 2014) and exposure to handling problems during transportation can lead to vaccine failure (Witter, 1998). Therefore, the development and production of effective vaccine to control the spread of the virus is crucial. Thus poultry farm owners, professionals and the veterinary service in the Ministry of Agriculture should give more attention on the prevention and control of Marek's disease (Berhan, 2014). This calls for development and production of effective MD vaccine locally.

Therefore the objectives of this study were:

- ✓ To isolate and molecularly characterize MDV from outbreak samples.
- ✓ To develop a trial vaccine against Marek's disease and evaluate its efficacy.

2. LITRATURE REVIEW

2.1. Disease Definition

Marek's disease (MD) is a lymphoproliferative and neuropathic disease of domestic chickens, and less common in turkeys and quails, caused by a highly contagious, cell-associated, oncogenic herpesvirus (Calnek, 1986; Schat and Nair, 2008; Sharma, 1998). 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).

2.2. 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* and genus *Mardivirus* (Marek's disease-like viruses).

MDV strains (MDVs) had become increasingly virulent since the 1960s (Witter, 1997; Osterrieder *et al*., 2006). MDVs were classified into three serotypes which had major differences in genome and biological features. Serotype 1 MDVs included all the oncogenic strains and their attenuated forms; serotype 2 MDVs were non-oncogenic viruses isolated in chickens; serotype 3 MDVs were non-oncogenic viruses isolated in turkey, generally known as herpesvirus of turkey or HVT (Bulow and Biggs, 1975).

2.3. MDV characteristics

Herpesvirus infectious particles comprise more than 30 different proteins, assembled according to a complex architecture including the following: (i) a central capsid containing the viral genome, (ii) a protein layer termed tegument, comprising more than 15 proteins, and (iii) a lipid bilayer in which about 10 envelope glycoproteins are anchored. The MDV genome is a linear double-stranded DNA of approximately 175 kb,

which contains a unique long (UL) sequence and a unique short (US) sequence, both flanked with terminal repeat (TR) and internal repeat (IR) sequences (Tulman, 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]); However, some genes are specific to MDV, such as the gene encoding Meq oncoprotein or pp38 phosphoprotein (Tulman, 2000).

The viral genome has the capacity to encode at least seventy proteins, sixty of which have counterparts in HSV, including structural proteins, metabolic enzymes and transactivating proteins such as VP 16 and ICP4 (34). However, at least ten MDV genes have no homologues in other herpesviruses. Similar to those of other herpesviruses, MDV genes also belong to three kinetic classes of immediate early, early and late genes, based on the requirements for viral protein synthesis and DNA replication. Compared to the extensive expression of genes, during the lytic infection, the transcription in latently infected and transformed cells has been largely restricted to the repeat regions of the MDV genome. Some of the important genes recognised within the repeat regions that could potentially be associated with transformation include the BamHI-H family transcripts, pp38, ICP4 sense and antisense ribonucleic acid (RNA) transcripts, and the meq gene.

2.3.1. Serotypes and pathotypes of MDV

Three serotypes of MDV and related Herpes viruses have been defined. Serotype 1 includes all the pathogenic or oncogenic strains of these viruses. Serotype 2 includes naturally non-attenuated strains of MDV. Serotype 3 includes turkey herpesvirus (HVT), the non-oncogenic MDV related virus isolated from turkey (table 1). New pathtypes have been emerging indicating continuous evolution of MDV towards greater virulence (Venugopal *et al*., 2001). Serotype 1 MDVs could be further classified into four

pathotypes, including mild (m), virulent (v), very virulent (vv) and very virulent plus (vv+) strains (Witter, 1983; Witter *et al*., 2005).

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

(Shambhu *et al*., 2012)

MDV infection results in the establishment of a latent infection, which is common in *Alphaherpesvirinae* ; however, MDV has unique properties when compared with other members of this virus subfamily because it can cause tumours and integrate its DNA into the host cell genome (Delecluse and Hammerschmidt, 1993; Delecluse *et al*., 1993).

3.3.2. Pathotypes and evolution of virulence

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

Thus, vvMDV are poorly protected by HVT but better protected by HVT + SB-1 vaccine. The vv+MDV are protected poorly by both vaccines (above), but CVI988/Rispens vaccine appears to offer somewhat better protection. Thus far, no antigenic differences have been noted among viruses of different Serotype 1 pathotypes, suggesting that the higher virulence associated with newer pathotypes is not likely due to antigenic drift. Some other heretofore unrecognized biological properties have been noted with vv+ strains, although it is not yet clear whether the changes are pathotype specific. Recently it has been noted that vv+ strains cause a particularly acute form of transient paralysis in 3-wk-old chickens, resulting in very high mortality (Witter, 1996).

Finally, Rosenberger and coworkers (1997) have noted instances in which recently isolated MDV strains of high virulence appeared to have increased pathogenicity for adult chickens, a finding consistent with that reported by Witter (1996)

The conditions that favor evolution of MDV towards greater virulence are not known but are probably created in part by vaccination with increasingly effective products. As

vaccinal immunity is compromised by factors such as early exposure or immunosuppressive stress, mutant clones have an increased opportunity to selectively multiply and to be seeded in the environment. Because vaccines are the major barrier to the disease, single point mutations may be sufficient to result in the emergence of a mutant strain (Witter, 1996).

2.4. Pathogenesis

The pathogenesis of MD is complex, with infection occurring throughout the respiratory route from inhalation of poultry house dust contaminated with the virus. After an early cytolytic infection mainly of the B-lymphocytes in the bursa, spleen and thymus, at 3 to 5 days post infection, the virus infects activated T-lymphocytes, mainly of the CD4+ phenotypes. The infection in the T-lymphocytes becomes latent at 6 to 7 days post infection and the virus is spread throughout the body by the infected lymphocytes that persist as a cell-associated viremia. A secondary cytolytic infection occur in the feather follicle epithelium form about ten days after infection, from where infectious cell-free virus is produced and shed into the environment in feather debris and dander (Murphy *et al*., 1999; Venugopal *et al*., 2001).

The lately infected T- lymphocytes are subsequently transformed leading to the development of lymphomatous lesions in visceral organs. The main target cells for transformation in natural infections are CD4+ T-cells, although the virus also has the potential to transform CD8+ T-cells (Murphy *et al*., 1999; Venugopal *et al*., 2001).

Birds infected with GaHV-2 can be carriers and shedders of the virus for life. Newborn chicks are protected by maternal antibodies for a few weeks. After infection, microscopic lesions are present after one to two weeks, and gross lesions are present after three to four weeks. The virus is spread in dander from feather follicles and transmitted by inhalation (Fenner *et al*., 2011).

2.5. Pathophysiology of Marek's Disease

The current model of MD pathophysiology was initially proposed by Bruce Calnek (Calnek, 1986; Calnek, 2001). This model is described in Figure 1. MDV enters via the chicken respiratory tract after inhalation of contaminated dust. Then MDV infects B lymphocytes and macrophages in the lungs (Baaten *et al*., 2009) and is then transported towards the main lymphoid organs (bursa of Fabricius, thymus, and spleen). After replicating in B lymphocytes, MDV infects activated T lymphocytes, mainly CD4+ cells. It is believed that only a few T lymphocytes undergo transformation and are at the origin of the T lymphoma, which may be either monoclonal or oligoclonal (Mwangi, 2011).

This lymphoma is mostly localized in visceral organs (kidneys, spleen, liver, gonads, and proventriculus), peripheral nerves, skin, and muscles. In most transformed T lymphocytes, the virus is in latent phase and does not produce viral particles. Only a small proportion of tumor cells (< 0.01%) expresses lytic viral antigens and contains viral particles detectable in transmission electron microscopy (TEM) (Rémy, 2013).

MDV only enters latency in lymphocytes but not in neurons, like most *alphaherpes-viruses*. Early during infection, the virus is transported towards the skin, most specifically to feather follicles. From infected feather follicles, MDV is shed into the environment via scales and feather debris, which become the major source of contamination of other birds in the natural environment. Bird-to-bird transmission is exclusively horizontal. There is no vertical transmission from the chicken to the egg, even though the embryo can be experimentally infected (St Hill, 2004).

In typical housing conditions, it is believed that animals become contaminated at a young age. MDV interactions with chicken skin is considered the major cause of MDV persistence in poultry houses and its evolution towards increasingly more virulent genotypes has been observed for the past decades (Gimeno, 2004; Nair, 2005).

Marek's disease virus (MDV) enters into the chicken through the respiratory tract (figure 1). MDV has a tropism for B- and T- lymphocytes as well as for the feather follicle epithelium, from which MDV is shedded into the environment. Feathers, skin danders and dust are the major source of MDV infectious materials and the basis of horizontal bird-to-bird transmission in field conditions (Calnek, 2001).

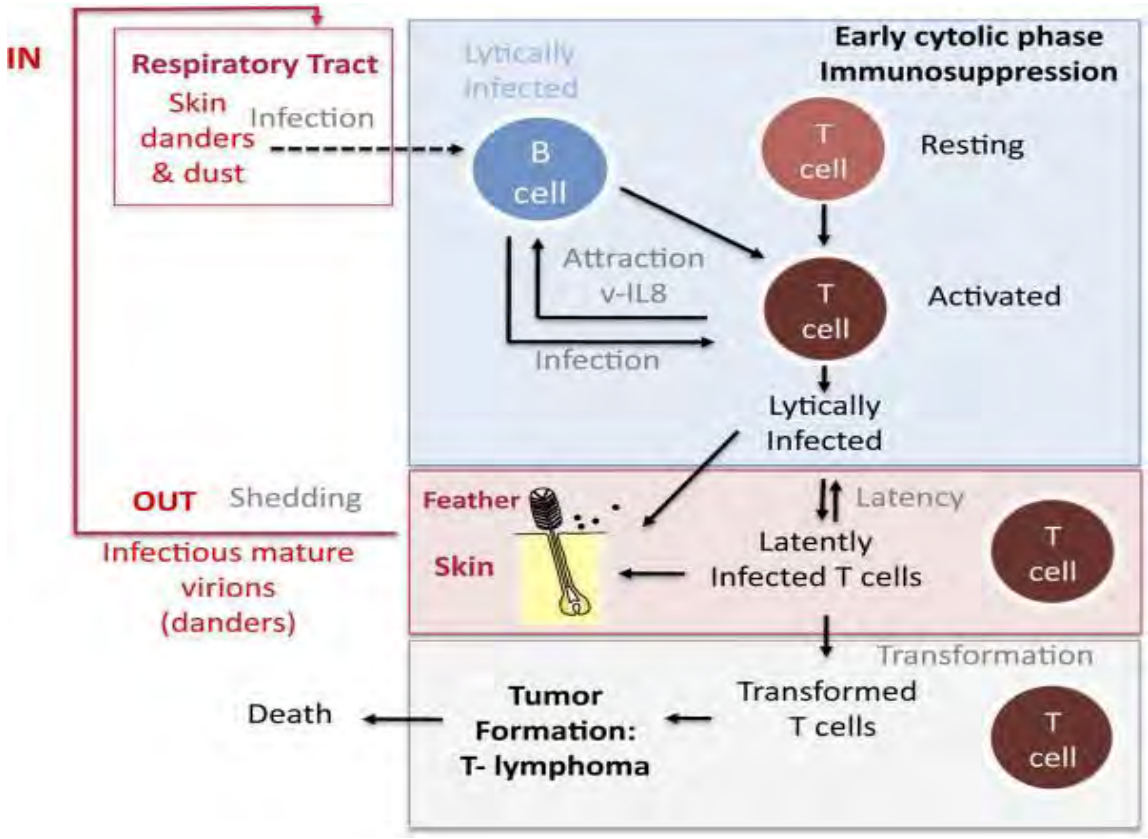


Figure 1: Pathophysiology of Marek's disease adapted from Calnek model.

2.6. Clinical Signs

There are six syndromes known to occur after infection with Marek's disease. These syndromes may overlap.

Classical Marek's disease or **neurolymphomatosis** in the classical form, the characteristic finding is enlargement of one or more peripheral nerves. Those most

commonly affected and easily seen at post-mortem are the brachial and sciatic plexuses, coeliac plexus, abdominal vagus and intercostal nerves (OIE, 2010).

Affected nerves are often two or three times their normal thickness, the normal cross-striated and glistening appearance is absent, and the nerve may appear greyish or yellowish, and sometimes oedematous. Lymphomas are sometimes present in the classical form of MD, most frequently as small, soft, grey tumours in the ovary, and sometimes also in the lungs, kidneys, heart, liver and other tissues. 'Grey eye' caused by an iridocyclitis that renders the bird unable to accommodate the iris in response to light and causes a distorted pupil is common in older (16–18 week) birds, and may be the only presenting sign (OIE, 2010).

Acute Marek's disease occurs in explosive outbreaks in young chickens, in which a large proportion of birds in a flock show depression followed, after a few days, by ataxia and paralysis of some birds. Significant mortality occurs without localizing neurologic signs. Visceral lymphomas are typically absent in affected birds, but nerve lesions are prominant (Fenner *et al*., 2011).

In the acute form, the typical finding is widespread, diffuse lymphomatous involvement of the liver, gonads, spleen, kidneys, lungs, proventriculus and heart. Sometimes lymphomas also arise in the skin around the feather follicles and in the skeletal muscles. Affected birds usually have enlarged peripheral nerves, as in the classical form. In younger birds, liver enlargement is usually moderate in extent, but in adult birds the liver may be greatly enlarged and the gross appearance identical to that seen in lymphoid leukosis, from which the disease must be differentiated. Nerve lesions are often absent in adult birds with MD (OIE, 2010).

Ocular lymphomatosis is a rare syndrome that leads to graying of the iris of one or both eyes as a result of infiltration of transformed lymphocyte; the pupil is irregular and eccentric, and there is partial or total blindness. Mortality is rare (Fenner *et al*., 2011).

Cutaneous Marek's disease is recognized readily after plucking round, nodular lesions occur at the feather follicles of young birds. The non-feathered area of the legs may have a distinct red coloration, and Marek's disease is therefore sometimes called "red leg syndrome" (Fenner *et al*., 2011).

Atherosclerosis is induced in experimentally infected chickens (Fabricant, 1999)

Immunosuppression impairment of the T-lymphocytes prevents competent immunological response against pathogenic challenge and the affected birds become more susceptible to disease conditions such as coccidiosis and "*Escherichia coli*" infection. Furthermore, without stimulation by cell-mediated immunity, the humoral immunity conferred by the B-cell lines from the Bursa of Fabricius also shuts down, thus resulting in birds that are totally immunocompromised (Islam *et al*., 2006).

2.7. Diagnosis

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 tumour cell, and (3) virological characteristics (Witter *et al*., 2010).

The demonstration of peripheral nerve enlargement along with suggestive clinical signs in a bird that is around three to four months old (with or without visceral tumors) is highly suggestive of Marek's Disease. Histological examination of nerves reveals infiltration of pleomorphic neoplastic and inflammatory lymphocytes. Peripheral neuropathy should also be considered as a principal rule out in young chickens with paralysis and nerve enlargement without visceral tumors, especially in nerves with interneuritic edema and infiltration of plasma cells (Bacon *et al*., 2001).

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)

In addition to gross pathology and histology, other advanced procedures used for a definitive diagnosis of Marek's disease include immunohistochemistry to identify cell type and virus-specific antigens, standard and quantitative PCR for identification of the virus, virus isolation to confirm infections and serology to confirm/exclude infections (OIE, 2010).

2.8. Marek's Disease Status in Ethiopia

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

On the other hand Duguma *et al.* (2005) investigated the prevalence, clinical and pathological manifestations and extent of mortality due to Marek's disease (MD) among indigenous chickens of Ethiopia reared under confined management at the Debre Zeit Agricultural Research Center, central Ethiopia. Clinical signs, mortalities, gross and microscopic examinations as well as virological and serological test results were used for the study. The study indicated an overall significant difference between manifestation of clinical signs in acute and chronic forms, respectively.

The extent and effectiveness of Marek's disease (MD) vaccination in reducing the incidence of mortality to natural MD challenge among 3 indigenous chicken ecotypes (Jarso, Tepi and Horo) together with Fayomi was also evaluated by Duguma *et al*., (2006). This study was conducted on indigenous chickens (Jarso, Tepi, Horro, and Fayoumi) chickens that were vaccinated against MD subcutaneously at neck region. Analyzed data by Duguma *et al.,* (2006) from daily records of mortality and survival of the chickens indicate that MD vaccination significantly increased the survival rate of Jarso, Horro and Fayoumi. The Tepi ecotype also showed improvement in survival rate due to MD vaccination but the improvement was not significant. This indicates the dramatic effect of MD vaccination on the survival of the indigenous chickens and it should be considered as an important management strategy while rearing them under confinement.

The results of the study made by Duguma *et al.*, (2006) indicated an overall significant difference between manifestation of clinical signs in acute (65.2 %) and chronic forms (34.8%), respectively. MD fatality rate was very high in this study where 97.9% diseased birds died. Serological prevalence study of MD using the Agar gel immuno-diffusion test (AGID) technique on 70 randomly sampled local chickens revealed an overall prevalence rate of 72.9 %. Information on circulating MD strains isolation in different production systems is also lacking. The studies summarized above indicate that MD is a serious problem for the poultry industry of Ethiopia and vaccination could be a vital instrument to combat against the threat. However, despite its growing importance of MD vaccine is being availed to the market by importation.

Study conducted by Berhan (2014) on isolation and molecular characterization of marek's disease virus from clinically diseased Ethiopian chickens reared under different production system; the result revealed that all Ethiopian marek's disease virus isolates were clustered under *Gallid Herpes virus* type 2.

Currently because of the high demand of vaccine by the private and government poultry production farms in the country, the National Veterinary Institute is importing marek's disease vaccine from India (INDOVAX PLC). Each dose of vaccine contains $\geq 10^3$ PFU of

HVT FC-126 Marek's disease virus strain. The vaccine strain is Meleagrid Herpesvirus 1, and this strain belongs to MDV serotype 3 or Herpes virus of turkeys and is a recommended vaccine strain since it is avirulent and non-oncogenic strain which is safer to birds and induces antibody production which can protect infection from other serotypes. Assessment of outbreaks in a country level and identification of the circulating virus type is very important to reach in a decision on the selection of safe and protective vaccine strain (Berhan, 2014).

2.9. Control and Prevention

2.9.1. Management factors to control MD

Following the introduction of HVT vaccine in 1971, losses from MD in broiler and layer chickens were dramatically reduced. Based on this early success, the poultry industry has relied on vaccination as the principal means of control. However, control can also be achieved through selection for host genes associated with resistance to tumor induction. Because MDV is not transmitted vertically, partial control may also be achieved through biosecurity procedures sufficient to delay exposure, such as placement of newly hatched chicks in thoroughly cleaned and disinfected houses that are well separated from houses with older chickens. Genetic selection and management have been used more frequently as adjuncts to vaccination rather than as primary control strategies but are critical components of an integrated control system (Davison and Nair, 2004).

Complete cleaning and disinfection after each crop because of the presence of the MDV in feather dander, it is important to remove all dust at clean-out. Maximum possible down-time (especially for broiler houses where birds are not vaccinated); multiple age flocks prevent proper clean-out. Know the origin of birds and don't mix birds from different sources. No visitors, maximum biosecurity. Only one type of poultry at each premise, Stress proper space and provision of feed and water (Schat, 2008).

2.9.2. Vaccine and vaccination

All the currently used vaccines are live vaccines derived from the three viral strains: the HVT FC126 strain (Okazaki, 1970), the GaHV-3 SB-1 strain, and the GaHV-2 CVI988/Rispens strain HVT and SB-1 vaccines are considered heterologous vaccines because they are derived from a different viral species than the virus it is intended to protect against, while the Rispens vaccine is considered homologous because it is from the same viral species as the targeted virus (Rispens *et al*., 1972).

Vaccination using HVT protected chickens against infections with vMDV, but failed to protect against newly emerging vvMDV in the late 1970s. Vaccines containing MDV-2 strains or combinations of HVT and MDV-2 were used successfully in the 1980s (Witter, 1997; Calnek *et al*., 1983), but within a few years novel vvMDV-1 strains such as 648A and 584A were isolated, which could break bivalent vaccination and cause acute transient paralysis as well as a significant increase in mortality within the first 2 weeks after infection. The appearance of vvMDV has led to the introduction of the CVI988/ Rispens vaccine (Witter, 1997).

The CVI988 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 (2004) the reasons for these possible failures include; challenge with virulent viruses before the development of vaccinal immunity; interference with the development of immunity by the maternal antibodies,; improper use of the vaccine; and the use of a non-protective vaccine strain.

Control of Marek's disease was predominantly via vaccination of chickens, three types of vaccine had been developed for use against MD. These were herpesvirus of turkey (HVT), non-pathogenic serotype 2 MDV and non pathogenic serotype 1. In 1970s, HVT vaccine was mainly used to control the disease. In mid-1980s, serotype 2 vaccine such as strain SB-1 was used in combination with HVT against the enhanced virulence strains. With

further increase in virulence of field viruses (Rispens *et al*., 1972), serotype 1 vaccine CVI988/Rispens (Rispens *et al*., 1972; De Boer *et al*., 1986) was introduced for widespread use in the 1990s.

2.10. Requirements for Vaccines

MD is prevented by vaccinating chickens *in-ovo* or at 1 day of age. Live viral vaccines are used. HVT (serotype 3), in either a cell-free (lyophilised) form, or a cell-associated ('wet') form, is one of the widely used vaccines. Attenuated variants of serotype 1 strains of MDV are the most commonly used vaccine. Serotype 2 strains may also be used, particularly in bivalent vaccines, together with HVT (OIE, 2010).

Serotype 1 and 2 vaccines are only available in the cell associated form. Bivalent vaccines consisting of serotypes 1 and 3 or trivalent vaccines consisting of serotypes 1, 2, and 3 are also used. The bivalent and trivalent vaccines have been introduced to combat the very virulent strains of MDV that are not well controlled by the monovalent vaccines. Vaccination greatly reduces clinical disease, but does not prevent the persistent infection by MDV. The vaccine viruses are also carried throughout the life of the fowl and are continued to be shed, which results in the ubiquitous presence of MDV (OIE, 2010).

Table 2: Licensed vaccine strains of MD (Witter, 1998)

Vaccine strain	Serotype
FC126 (HVT)	3
SB-1	2
301B/1	2
CVI988 clone C	1
CVI988/C/R6	1
CVI988 (Rispens)	1
R2/23 (Md11/75)	1

2.11. Vaccination against Marek's Disease

2.11.1. Attenuated serotype 1 MDV Vaccines

Attenuated virulent serotype 1 vaccines

The first effective vaccine against MD was developed by Churchill *et al*., (1969a,b) following the discovery that the oncogenic HPRS-16 MDV isolate could be attenuated by passage in cell culture and that the attenuated virus protected chickens against MD. The attenuated HPRS-16 vaccine was used extensively and considered effective, but has now been superseded by other vaccines. Reversion to virulence and spread has not been reported.

Attenuated mildly virulent serotype 1 vaccines

Rispens *et al*., (1972 a,b) described a vaccine derived from strain CVI-988, a mildly virulent serotype 1 MDV isolate which was further attenuated by 20 cell culture passages. This vaccine has been used against MDV strains of very high virulence. The vaccine has been shown to be mildly virulent for highly susceptible lines of chickens and maintains its ability to spread by contact (Rispens *et al*., 1972a; von Bulow, 1977).

Attenuated serotype 1 vaccines derived from highly virulent strains

Witter (1982) described an attenuated very virulent strain, Md11/75C, which was considered effective against challenge with very virulent MDVs. However, it was readily neutralized *in vivo* and was relatively ineffective in chickens with maternal antibodies (Witter and Lee, 1984). The vaccine virus was also shown to be partially virulent after back passage in chickens.

2.11.2. Serotype 2 MDV vaccines

The protective efficacy of serotype 2 viruses was first observed by Zander *et al*., (1972) in vaccination experiments which were later found to contain the serotype 2 strain HN-1. The SB-1 strain (Schat and Calnek, 1978) is used and has been shown to be non-oncogenic since no proliferative lesions have been observed. Jackson *et al*., (1977) used a naturally non-oncogenic MDV-19 strain as a vaccine.

2.11.3. Serotype 3 MDV vaccines

The Herpesvirus of turkeys (HVT; Serotype 3 viruses) are non pathogenic for turkeys and chickens, and were first identified in turkey kidney cell cultures by Kawamura and co-workers in 1969. Since their discovery and commercial propagation, serotype3 viruses, and in particular, the FC126 strain (Witter *et al*., 1970) has been widely used throughout the world, as a cell- associated form of vaccine against MD. A cell-free form of vaccine also exists but is less protective than its cell-associated counterpart (Witter, 2001).

2.11.4. Polyvalent vaccines

Better protection from MD was found when combinations of serotypes were used together in a vaccine than either serotype alone. Termed 'Protective synergism', it is unique to MD and is strongly serotype specific. It was demonstrated between the SB-1 strain (serotype 2) and FC126 strain (HVT) (Schat *et al*., 1982; Witter, 1982), which stimulated the development of the first commercial vaccine based on this phenomena between serotype 2 and 3 viruses (Schat *et al*., 1982, Witter, 1982). Synergism is especially evident between serotypes 2 and 3 but seems to be less pronounced with other combinations. With the recent introduction of serotype 1 vaccine, trivalent combinations of all three serotypes have been used. Trivalent vaccines FC126 + 301/B + CVI988/C (serotypes 1, 2 and 3 respectively) and FC126 +SB-1 + CVI988/C were introduced in 1990 and are currently recommended only for high risk flocks.

2.11.5. Recombinant vaccines

There have been numerous attempts to develop recombinant vaccines against MD using HVT (Ross *et al*., 1993), fowlpox (Heine *et al*., 1997; Nazerian *et al*., 1996) and Newcastle disease (Morgan *et al*., 1992,; Sonoda *et al*., 1996; Reddy *et al*., 1996) viruses as vectors but none have proved as efficacious as the standard HVT vaccine.

Based on estimates derived from comparison of actual condemnation of chickens and predicted losses in the absence of vaccination, vaccine efficacy is extremely high, although the efficacy of MD vaccines presented in table 3 as it is viewed by (Witter, 1998).

Table 3: Most common vaccine formulations for Marek's disease (MD)

Most common vaccine Formulations	Relative efficacy vs MDV pathotype		
	V	VV	VV+
FC126 alone	+++	+	+
FC126 plus Serotype 2	+++	+++	++
CVI988 (Rispens) alone or plus Serotype 3 or plus Serotypes 2 and 3	+++	+++	+++

2.12. Rationale and Intended use of MD Vaccine

Control of MD is essentially achieved by the widespread use of live attenuated vaccines (Nair, 2004). Commercial biological products used in the control of MD are the cell-associated or cell-free (lyophilised) live virus, respectively. Marek's disease vaccines are injected *in ovo* at the 17^{th} or 18^{th} day of embryonation or subcutaneously at hatch (Sharma, 1999).

2.13. Development of Marek's disease challenge viruses

Marek's disease vaccines have been evaluated by challenging immunized chickens with virulent Strain of serotype 1 viruses and observing for deaths, morbidity and typical lesions over a predetermined period, generally from 6 to 10 weeks (Biggs and Milne, 1972; Witter, 1991a).

Birds that die during the period and those that survive are subjected to post-mortem examination and assessed for the presence of tumours, nerve and other MD-specific lesions. Many MD challenge viruses consist of preparations of lymphocytes from birds experimentally infected with a virulent strain of Marek's disease virus (MDV), however this procedure is both time consuming and expensive. It requires the use of chickens and appropriate holding facilities and introduces significant risks from spread of the disease to other susceptible birds. Many of these disadvantages can be overcome using challenge viruses prepared and assayed in cell culture (Okazaki *et al.*, 1971; Payne and Rennie, 1973; Witter, 1982).

However, passage in cell culture has been shown to be associated with a reduction of virulence (Churchill *et al*., 1969a; Konobe *et al*., 1979; Witter, 1991a) and the extent of this reduction varies according to experimental conditions. Churchill *et al*. (1969a) described a loss of pathogenicity for chickens. Field isolates of MDV pass a limited number of times in chicken embryo kidney (CEK) and chicken embryo fibroblast (CEF) cultures prepared from specific-pathogen-free (SPF) embryos and shown to retain their virulence. Preparations of each strain could be readily stored as seed-lots and assayed by standard techniques (OIE, 2010).

3. MATERIALS AND METHODS

3.1. Study Area

The study was conducted from November 2014 to May 2015 in six selected areas of central Ethiopia, based on MD outbreak report. Samples were collected from Addis Ababa, Akaki, Dukem, Bishoftu, Mojo and Adama poultry farms which were purposively included in the present study since MD outbreaks were occurred during the study period. These areas were known as the main belt for poultry production in Ethiopia (Figure 2).

Study areas Addis Ababa, Akaki, Dukem, Bishoftu, Mojo and Adama are located in central Ethiopia at latitude range from 8°53'N to 9°03N , longitude 38°74'E to39°27E and daily average temperature ranging from 9.9 to 34ºC with mean annual rain fall of 1224 mm and elevated 2110 above sea level (Tesfaye, 2009). Dukem and Bishoftu towns are located 40 and 47 km respectively, while Mojo 88 km and Adama 99 km distant south east of the capital city.

The laboratory investigation on outbreak collected samples and vaccine development trials were conducted at the National Veterinary Institute (NVI) located at Bishoftu.

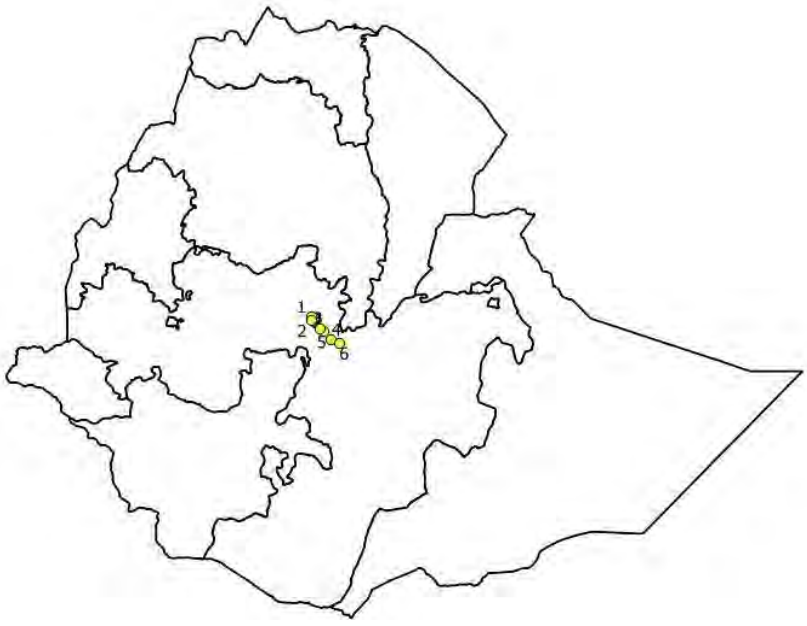


Figure 2: Map of Ethiopia showing the study areas where outbreak MD samples were collected from chickens. Where 1-Addis Ababa, 2-Akaki, 3-Dukem, 4-Bishoftu, 5-Mojo, and 6-Adama.

3.2. Study Animals

Outbreak investigation was conducted in a total of 18,661 chickens, where 16,836 female layer chickens and 1,825 male broiler chickens in six poultry farms. From 2514 clinically sick chicknes 25% of chickens (n=628) were examined. Primary data were collected from farm supervising veterinarians and farm owners using questionnaire. To evaluate the challenge virus pathogenecity and trial vaccine immunogenicity a total of 200 male day-old, Bovans brown chickens were used.

3.3. Experimental Design

A total of 200 male, Bovans brown chickens were used. For MD challenge virus pathogenicity validation test 130 chickens were grouped using complete random design in to 7 groups (6 test and 1 control groups). For vaccine evaluation 70 chickens in seven experimental groups (6 test and 1 control groups) were assigned randomly. The procedure followed was basically described in Experiment 1 and 2. All groups were housed in separate classes with wire mesh partition. Experimental chickens were followed for a periods of 10 weeks post-challenge. Chickens that died or were euthanized at the end of the experiment were examined for evidence of tumours (Celina et al., 2004).

3.3.1. Experiment 1

Complete random design method was used to assign a total of 130 male day-old Bovans brown chickens in seven experimental groups to validate the pathogenecity of local MDV isolates using Witter model (Witter, 1997). In the first six groups 120 chickens were assigned in 20 chickens per group and 10 chickens for control group. In each of the first six experimental groups 10 chickens per group were inoculated through intra-peritoneal route at a dose of 1000 Plaque Forming Units (PFU) and the rest 10 chickens per group were inoculated with 500PFU of challenge virus and identified with black colour, permanent marker at the head (renewed every week) and red plastic ring was tighten on their leg. Chickens were inoculated with the appropriate MD challenge virus of field

isolates from Addis Ababa (1), Akaki (2), Dukem (3), Bishoftu (4), Mojo (5) and Adama(6) in respect of their group. The code of each isolates was given for the purpose of this study only. In group seven 10 chickens were left unchallenged and used as a negative control; these chickens were treated apart from the other challenged groups in a separate house. All groups were followed for 10 weeks post inoculation. Chickens that were died during the observation period and killed at the end of observation were examined for gross lesions (Gimeno *et al*., 2008). lesions scored for liver, spleen, heart and lung tumorous nodules (pin-point to 2 mm in diameter), grayish-white in colour which were firm in consistency were considered as positive (Kamaldeep *et al*., 2007). The result obtained from this was used as an evidence to evaluate the pathogenic nature of MDV isolates.

3.3.2. Experiment 2

A total of 70 male day-old, Bovans brown chickens were assigned in seven experimental groups to evaluate trial vaccine efficacy. In the first six groups 60 chickens were assigned in 10 chickens per group. In each of the first six experimental groups chickens were immunized with trial Vaccine formulation (HVT FC 126) at a dose of 2000PFU per chicken subcutaneously at 1 day of age (Gimeno *et al*., 2008). Immunized chickens were challenged through intra-peritoneal route at 8 day of age with 1000 PFU/chicken with the appropriate challenge virus of field isolates. Chickens were observed for 10 weeks post-challenge (Celina *et al*., 2004). The results obtained from these groups were compared with the result of experiment 1 challenged groups with 1000PFU per chicken and used as a positive control with their respective local challenge virus isolates. 10 sentinel chickens which were immunized with trial Vaccine at a dose of 2000PFU per chicken subcutaneously at 1 day of age and were left for negative control.

3.4. Field Clinical Examination

A total of 18661 chickens from six study area of eight poultry farms in central Ethiopia were examined for MD clinical sign. However, from a total of 2514 clinically sick chickens 628 chickens were purposively selected and thoroughly examined. Physical

examination focused on clinical signs of depression; loss of weight, Skin around feather follicles raised and roughened, flaccid neck, paralysis of wing and leg.

3.5. Questionnaire Survey

The questionnaire survey was carried out in poultry farms where MD outbreak occurred. Farm supervisor veterinarian and farm owners were purposively selected. A total of 8 respondents were interviewed using semi-structured questions for epidemiological data regarding total chicken, number of affected birds, number of birds reported dead due to MD, farming system age, breed, sex, clinical findings, vaccination status, were collected from all poultry farms. Clinical examination result and post mortem findings were recorded in the prepared format (Annex 12).

3.6. Outbreak Sample Collection and Transportation

Marek's disease outbreak report based, purposive sampling strategy was used for Sample collection. Samples were collected from clinically sick chickens. History of the farm and basic information about the disease was recorded to rate mortality and morbidity in respect of vaccination history. Clinical samples were collected following careful examination of individual cases. For virus isolation tissues of liver, spleen, lung and feather follicle were collected aseptically from MD suspected clinically sick chickens and post mortem findings were recorded. Samples collected from the field were transported using cold-chain to the diagnostic laboratory of NVI. In order to maintain the viability of virus, samples were stored at -80ºC until laboratory analysis carried out (Witter, 1997; OIE, 2010).

3.7. Laboratory Investigation

3.7.1. Cell culture

Primary chicken embryo kidney (CEK) cells were prepared from 18-day old embryonated specific pathogen free (SPF) eggs. Primary chicken embryo fibroblast (CEF) cell was also prepared from 10 - 11 day Old embryonating SPF eggs for the isolation of MDV (OIE, 2010).

3.7.2. Virus isolation

MD virus was detected by isolating the virus from the suspected tissue samples as stated in Schat *et al*., (1982). Accordingly, 10% w/v tissue suspensions were prepared using sterile phosphate buffer saline (PBS) and inoculated onto monolayer cultures of CEK grown in plastic cell culture flasks for four consecutive passages and then inoculated to CEF cell again up to four consecutive passages. Inoculated and non-inoculated control cultures were incubated at 37°C in a humid incubator containing 5% CO_2. Culture medium was replaced at every 2-day intervals. Areas of cytopathic effects (CPE) were appreciated within 6–8 days of incubation. Blind passage was conducted using freeze and thaw of the cultures in all consecutive passages (OIE, 2010).

3.7.3. Virus DNA extraction

Virus DNA was extracted from the tissue suspension and CPE positive cell culture homogenate using DNeasy® Blood and Tissue Kit (QIAGEN, Germany) following the manufacturer's instruction in the facilities of the molecular biology of the National Veterinary Institute (NVI). The eluted virus DNA was kept at -20ºC in a labelled eppendorf tube. Details of the steps to be followed for DNA extraction are described in Annex (6).

3.7.4. PCR amplification and Agarose gel electrophoresis

PCR offers faster and more reliable test as diagnostic tool of Marek's disease (Handberg *et al.*, 2001; Islam *et al.*, 2004). PCR was performed targeting the amplification of the ICP4 gene of MD virus (318bp) using

Forward primer M1.1 5'- GGATCGCCCACCACGATTACTACC-3' and

Reverse primer M1.8 5'-ACTGCCTCACACAACCTCATCTCC-3'

The PCR was carried out in a final reaction volume of 20 µL using 200 µL capacity thin wall PCR tube containing 4 µL template DNA, 10× PCR buffer, 25 mM $MgCl_2$, 200 µM of the four dNTPs, 10 pmol of each primer, and 1U Taq DNA polymerase. The PCR tubes with all the components were transferred to a thermal cycler (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 for 1min, annealing 55°C for 10s and extension 72°C for 1min, and final extension at 72°C for 7 min. PCR products were loaded and separated using electrophoresis apparatus (BIORAD) at 100 Volts for 60 min in 2% Agarose gel stained with ethidium bromide (OIE, 2010). The gel was visualized through UV trans-illuminator gel documentation system and gel picture was captured using a Polaroid photograph camera (UVitec) and results were recorded.

3.8. Challenge Virus Preparation and Assay

Marek's disease outbreak samples were isolated from flocks in central Ethiopia that had been experiencing high MD mortalities. Each strain was first isolated in tissue culture of Chicken Embryo Kidney (CEK) cell. Those that had not showed CPE were blindly passaged up to the fourth passage and then passed to chicken embryo fibroblast (CEF) cells. Each of the six isolates was suspended in Cell Freezing Media (CFM) and stored at -20^0C (OIE, 2010). Each isolates were inoculated in chickens to confirm their pathog-enicity and used as a challenge virus at the 8^{th} passage and tested for their identity using PCR.

The challenge virus titer was determined by the Tissue Culture Infective Dose (TCID 50) assay method indicated in annex (4) using Spearman Karber method and the result was converted to Plaque Forming Unit (PFU) [assuming 1 TCID50 = 0.69 PFU (Luria *et al*., 1978)]. The six field isolates selected for pathogenicity and challenge study were prepared in 1000 and 500PFU/dose as per the sample titer result.

3.9. MD Vaccine Preparation and Dose Formulation

The trial vaccine was prepared by cultivating the virus, HVT FC 126 strain, on Chicken Embryo Fibroblast monolayer cell, prepared from 11days embryonated SPF egg. At the beginning of a production run, working seeds were prepared from the master seed stock virus, originated from India, in CEF cell cultures and then production seeds were prepared from working seeds. The titer was determined by the Tissue Culture Infective Dose (TCID 50) assay method and the result was converted to Plaque Forming Unit (PFU) [assuming 1 TCID50 = 0.69 PFU (Luria *et al*., 1978)]. One dose of the vaccine was formulated to the equivalent of 2000 PFU/0.2ml in Cell Freezing Media. The production seed viruses were tested for purity, identity and titer (OIE, 2010). General manufacturing details were described in annex (3).

3.10. Statistical analysis

The collected data during sampling and laboratory analysis were coded and stored into Microsoft office Excel spread sheet 2007. The data were thoroughly screened before statistical analysis. Descriptive statistics at 95% confidence interval was used to summarize data of lesion score, questionnaire and laboratory findings using Stata sa 11 soft. For individual study, chickens were divided into two categories (lesions and no lesions) based on the presence or absence of MD lesions at death of the chicken or at termination of the study. For vaccine efficacy, treatment groups were divided into two categories (protected [p] and non-protected [np]) based on an arbitrary criterion 25% cut off point (Gimeno *et al*., 2008).

4. RESULTS

4.1. Questionnaire Survey

From a total of 18661 chickens 2514 (13.5%) were sick, 1072 (5.7%) were dead and 15075 (80.8%) were at risk. Chickens from 3-6 months of age (87.4%) were more susceptible than other chickens < 3 months of age (9.8%) and >6 months of age (2.8%).

Table 4: Summary of questionnaire survey

Sample area	Production type	Age	No. Sick (% morbidity)	No. Death (% mortality)	No. at risk	Vaccination history
Addis Ababa (kolfe)	layers	5.5 month	15	2	435	Vaccinated (HVT Fc126)
Bishoftu	layers	5 month	1500	594	2400	Unvaccinated
Dukem	layers	1 year	5	1	500	Vaccinated (HVT Fc126)
Akaki	layers	6 month	104	60	40	Unvaccinated
Dukem	layers	5 month	800	400	2400	Unvaccinated
Bishoftu	broilers	20 days	20	5	1800	Vaccinated (HVT Fc126)
Mojo	layers	5 month	30	0	1500	Vaccinated (HVT Fc126)
Adama	layers	3 month	40	10	6000	Vaccinated (HVT Fc126)
	Total		**2514 (13.5%)**	**1072 (5.7%)**	**15075**	

4.2. Mortality and Morbidity

MD event of mortality and morbidity rate based on vaccination history of Marek's disease outbreak was summarized in table 5.

Table 5: Morbidity and mortality rate of MD outbreak in vaccinated and unvaccinated flocks

	Morbidity /Mortality (%) in individual farm							
Event	1	2	3	4	5	6	7	8
Vaccinated chicken	3.3/0.4		1/0.2			1/0.3	1.9/0	0.7/0.2
Un vaccinated chicken		33.4/13.2		51/29.4	22.2/11.1			

Morbidity in vaccinated farms ranges from 0.7% to 3.3% and in unvaccinated farms it ranges from 22.2% to 51%. Mortality in vaccinated chickens range from 0.2% to 0.4% and in unvaccinated chicken it ranges from11.1% to 29.4%. This result implied that vaccination has reduced the effect of MD.

4.3. Clinical Examination

From 628 clinically examined chickens, 407 chickens (64.8%) developed paralysis of leg and wing. 221 chickens (35.2%) developed other clinical signs depression, loss of appetite, weight loss, difficulty in breathing, and death (figure 3). From 407 paralyzed chickens 396 (97.3%) were occurred in unvaccinated and 11 (2.7%) in vaccinated chickens. This implied that vaccination has been reduced the occurrence of paralysis.

Table 6: Chickens clinically examined and Post mortem finding summary and sampled

Sample area	No. examined	Paralysis	Vaccination history
Addis Ababa (kolfe)	4	3	Vaccinated (HVT Fc126)
Bishoftu	375	263	Unvaccinated
Dukem	2	2	Vaccinated (HVT Fc126)
Akaki	26	16	UN
Dukem	196	117	Unvaccinated
Bishoftu	6	2	Vaccinated (HVT Fc126)
Mojo	9	1	Vaccinated (HVT Fc126)
Adama	10	3	Vaccinated (HVT Fc126)
Total	628	407	

Chickens that were examined for post mortem showed enlarged spleen and liver, necrotic foci on heart and lung in most examined MD suspected chickens from poultry farms of Addis Ababa, Akaki, Dukem, Bishoftu, Mojo and Adama.

Figure 3: Clinical signs findings of Marek's disease suspected chickens showing (A) depression, (B) flaccid neck, (C) paralysis of wing, (D) paralysis of leg

4.4. Post mortem Findings

Post mortem examination result revealed white foci of neoplastic tissue in liver, heart and lung, enlargement of spleen in most cases were observed. From a total of 13 post mortem chicken samples lesion were found in 12 chickens (92%) and 1non lesion chicken (8%).

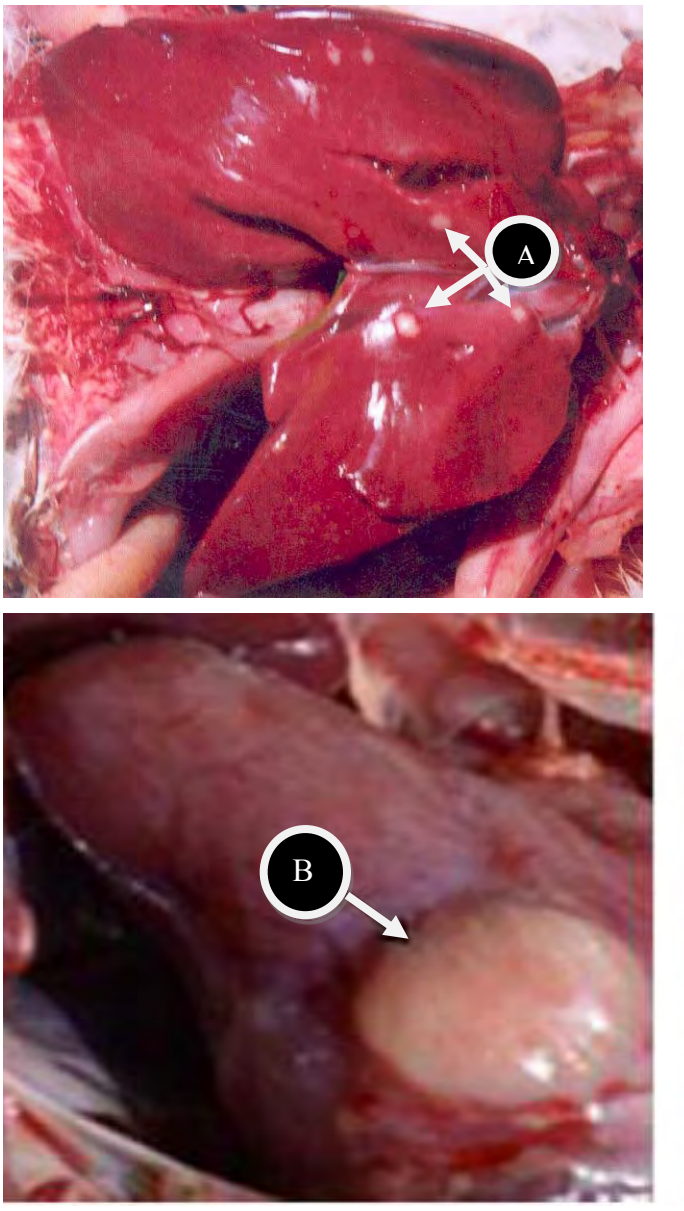


Figure 4: Postmortem findings from Marek's disease suspected chickens
(A) enlarged size and white arrow indicates tumors in liver and
(B) white arrow indicating tumor in heart

4.5. Outbreak Sample Laboratory Result

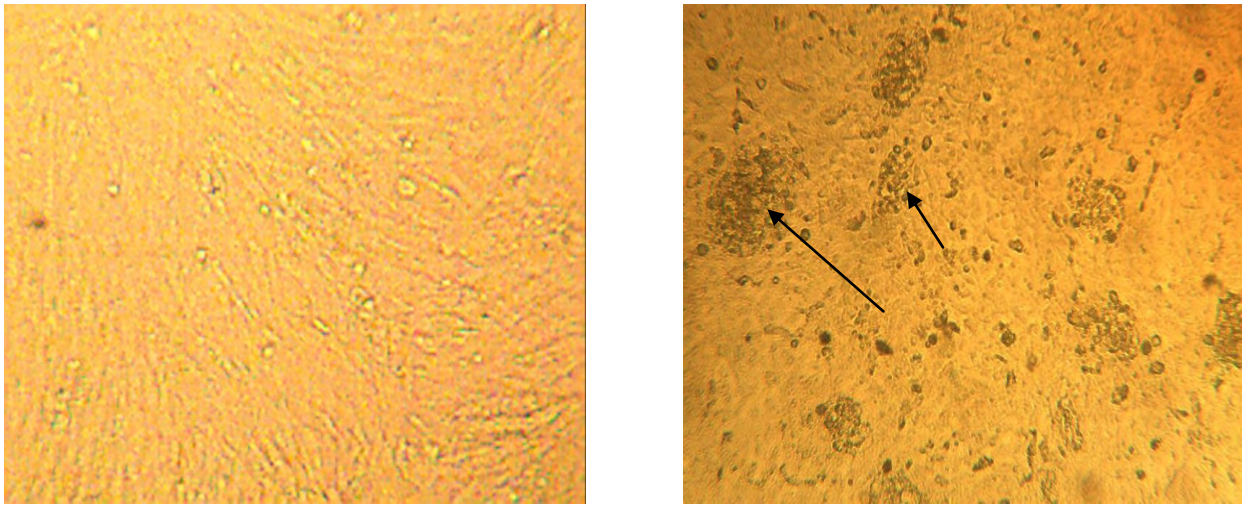
Table 7: MD outbreak samples laboratory test result

No.	Sample area	Sample type	Sampled tissue/pool	Test method	Test result per pooled sample			
					Positive (%)		Negative (%)	
1	Addis Ababa	Spleen	3/1	Cell culture, PCR	1	(100)	0	
		Liver	3/1	Cell culture, PCR	1	(100)	0	
		Lung	3/1	Cell culture, PCR	1	(100)	0	
2	Akaki	Spleen	3/1	Cell culture, PCR	1	(100)	0	
		Feather	5/5	PCR	4	(80)	1	(20)
3	Dukem	Spleen	3/1	Cell culture, PCR	1	(100)	0	
4	Bishoftu	Spleen	3/1	Cell culture, PCR	1	(100)	0	
		Feather	5/5	PCR	4	(80)	1	(20)
5	Modjo	Liver	2/1	Cell culture, PCR	1	(100)	0	
		Spleen	2/1	Cell culture, PCR	1	(100)	0	
		Feather	5/5	PCR	5	(100)	0	
6	Adama	Spleen	2/1	Cell culture, PCR	1	(100)	0	
		Liver	2/1	Cell culture, PCR	1	(100)	0	
		feather	5/5	PCR	5	(100)	0	
Total			46/30		28	(93.3)	2	(6.7)

Samples positive in cell culture were pooled in their respective sample types and sample area and prepared to run PCR. The result revealed that from a total of 46/30 pooled collected samples 28 samples 93.3% were positive for MD and 2 samples 6.7% were found negative.

4.6. MDV Isolation

MD positive sample by laboratory test was cultured to CEK and CEF cell and passaged to appreciate CPE. The result revealed that an early CPE was seen as small round cells reflecting light waves. These cells formed foci and syncytia later that detached from the wall of cell culture flask causing formation of plaque (figure 5).



(A) CEF monolayer cell after 48 hrs incubation (B) MDV CPE, passage 8 at 4th day,

Figure 5: MD virus growth on chicken embryo fibroblast cells. where (A) Confluent monolayer of chicken embryo fibroblast (CEF) cell grown in GMEM growth medium after 48 hours of culture; (B) Characteristic cytopathic effect (CPE) of MD virus (black arrow) on CEF cell developed after 4th days post-infection of passage 8.

4.7. Detection of viral DNA by Conventional PCR

Polymerase chain reaction was performed targeting to amplify a 318bp fragment of ICP4 gene of MDV using the forward primer M1.1 and reverse primer M1.8 to reach a definitive diagnosis of the causative agent of the outbreak. Six wild strain MD virus isolates were tested for MDV genome. All samples showed the presence of 318bp PCR products (Figure 6).

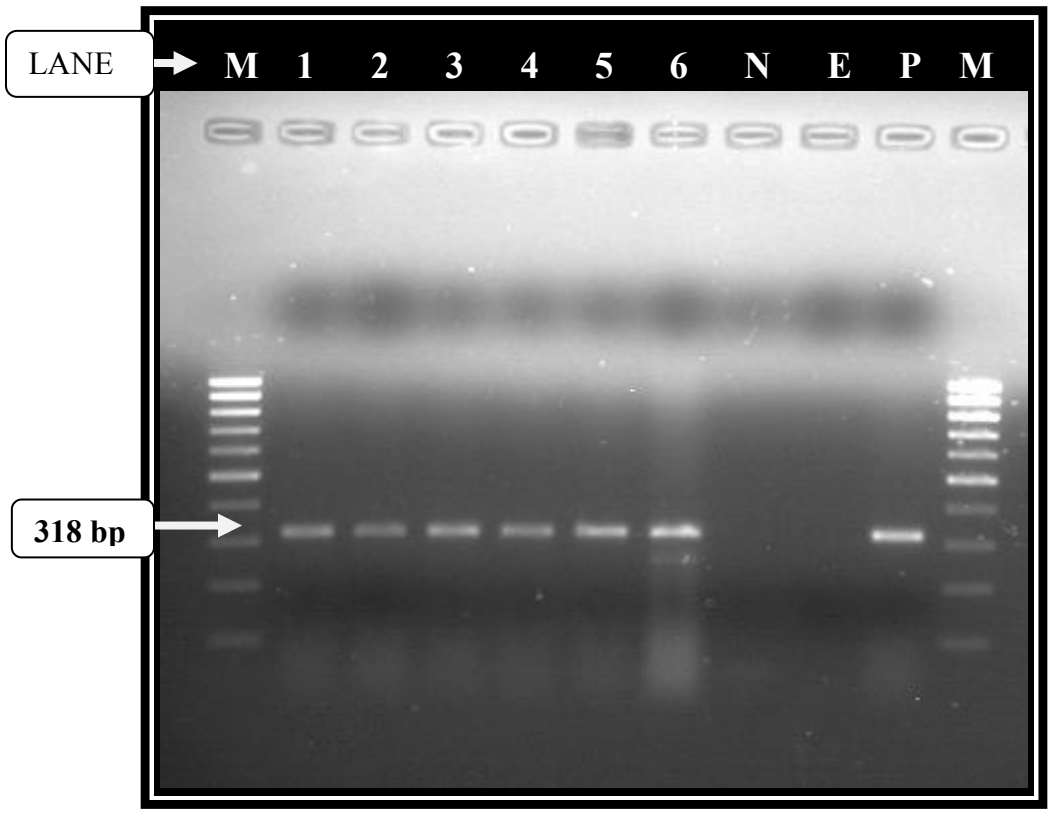


Figure 6: Gel electrophoresis of 318 bp PCR product of challenge MDV

Where M- Molecular marker started at 100bp (Fermentas), 1- 6 Challenge MDV at passage-8 positive around 318bp: 1-Addis Ababa isolate, 2-Akaki isolate, 3-Dukem isolate, 4- Bishoftu isolate, 5- Mojo isolate, and 6- Adama isolate. N negative controls without template- no amplification. E RNase free water extraction control- no amplification. P positive control - positive around 318bp (known MD tissue suspension sample)

4.8. Challenge Virus and Trial Vaccine Titration Result

Table 8: Result of Challenge virus and trial MD vaccine titration

MD virus Titration	Titer per ml in		Challenging and vaccination dose in PFU	
	TCID 50	PFU		
01	$10^{3.7}$	3458	500 (0.15ml)	1000(0.30ml)
02	$10^{3.8}$	4353	500 (0.12ml)	1000(0.24ml)
03	$10^{3.8}$	4353	500 (0.12ml)	1000(0.24ml)
04	$10^{3.7}$	3458	500 (0.15ml)	1000(0.30ml)
05	$10^{3.8}$	4353	500 (0.12ml)	1000(0.24ml)
06	$10^{3.8}$	4353	500 (0.12ml)	1000(0.24ml)
HVT FC 126 vaccine	$10^{4.2}$	10935	2000 (0.2ml)	-

variable	Mean	Std. Err.	[95% Conf. Interval]	
PFU	4054.667	188.6826	3569.643	4539.691

The mean titration result of the six MD challenge virus was 4054.67 PFU per ml and the trial vaccine formulation from HVT FC 126 was 10935 PFU per ml. The dose of injection per chicken was determined depending on their respective titer value to evaluate the pathogenicity of challenge MDV. Although in this experiments no reference strains were used for comparative purposes, but the two alternative doses (500 and 1000 PFU) of all isolates were evaluated for virulence of the virus and evaluated to each other.

4.9. Validation of Local Isolate MD Challenge Virus

Table 9: Validation of MD challenge virus in two alternative doses

Experiment	Group size	Breed	Challenge virus used							
			Local isolate	Alternative 1				Alternative 2			
				Dose /PFU/	Death	lesion	MD positive	Dose /PFU/	Death	lesion	MD positive
1	20	Bovans brown	01	500	0/10	8/10	80%	1000	4/10	6/10	100%
	20	Bovans brown	02	500	0/10	7/10	70%	1000	5/10	5/10	100%
	20	Bovans brown	03	500	0/10	7/10	70%	1000	3/10	7/10	100%
	20	Bovans brown	04	500	0/10	8/10	80%	1000	4/10	6/10	100%
	20	Bovans brown	05	500	0/10	6/10	60%	1000	3/10	7/10	100%
	20	Bovans brown	06	500	0/10	7/10	70%	1000	4/10	6/10	100%
	10	Bovans brown	control	-	-	-		-	-	-	-

Variable	Obs	Mean	[95% Conf. Interval]	
Alternative1	6	71.67	63.77	79.57
Alternative2	6	100	100	100

In both alternatives, challenged chickens were positive for MD. However, in alternative 1; chickens were not dead within study period and lesion score observed 60-80% with a mean of 71.67% MD incidence. In alternative 2 death was observed from 30% -50% and lesion from 50%-70% with a total of 100% MD positive result was recorded with a mean of 100%. Statistically there

was a significant difference between the two alternatives. The overall result implied that there all isolates were pathogenic and were not lost their virulence during passage. Therefore all isolates were capable to induce MD at a dose of 1000 PFU.

4.10. Immunization and Challenge Test

Table 10: Pathogenicity of local isolate MD virus and protective efficacy of Trial vaccine

Experiment	Challenge virus	Chicken breed	Vaccine	* MD incidence Positive/total (%)		Total	***Protective index
				Mortality	**PM lession		
2	01	Bovans brown	HVT FC 126	0/10(0)	1/10(10%)	1/10(10)	90
	02	Bovans brown	HVT FC 126	0/10(0)	0/10(-)	0/10(-)	100
	03	Bovans brown	HVT FC 126	0/10(0)	1/10(10%)	1/10(10)	90
	04	Bovans brown	HVT FC 126	0/10(0)	0/10(-)	0/10(-)	100
	05	Bovans brown	HVT FC 126	0/10(0)	0/10(-)	0/10(-)	100
	06	Bovans brown	HVT FC 126	0/10(0)	1/10(10%)	1/10(10)	90
Negative control	-	Bovans brown	HVT FC 126	0/10(0)	0/10(-)	0/10(-)	100

Variable	Obs	Mean	Std. Err.	[95% Conf. Interval]	
Protective Index	6	95	2.236068	89.252	100.748

* Chicken vaccinated at 1 day old and challenged at 8 days old; MD incidence (mortality plus survivors with lesions) determined during the post-inoculation period of 10 weeks.

** Post mortem lesion- Tumours do not included in birds that died during experiment period

*** Protective Index (PI %) = $\frac{\text{\% MD Positive control - \% MD observed group}}{\text{\% MD Positive control}}$ X 100 (Gilmore and Tannock, 2004)

The mortality and post mortem lesion score after challenge was recorded and MD incidence was rated from 0-10% with a mean MD incidence rate of 5% in experiment 2. The protective index ranges from 90% to 100% with mean value of 95%. This implied that chickens immunized and challenged were protected against MD as compared to experiment 1 result.

Table 11: Actual MD incidence after challenge

Experiment	Group size	Breed	Vaccine used				Challenge virus used				*Expected MD %	**Actual MD%
			strain	Dose in PFU	Age /days/	R/A	Local isolate	Dose in PFU	Age /days/	R/A		
2	10	Bovans brown	HVT FC126	2000/0.2ml	1	Sc	01	1000	8	Ip	<25	10
	10	Bovans brown	HVT FC126	2000/0.2ml	1	Sc	02	1000	8	Ip	<25	0
	10	Bovans brown	HVT FC126	2000/0.2ml	1	Sc	03	1000	8	Ip	<25	10
	10	Bovans brown	HVT FC126	2000/0.2ml	1	Sc	04	1000	8	Ip	<25	0
	10	Bovans brown	HVT FC126	2000/0.2ml	1	Sc	05	1000	8	Ip	<25	0
	10	Bovans brown	HVT FC126	2000/0.2ml	1	Sc	06	1000	8	Ip	<25	10
	10	Bovans brown	HVT FC126	2000/0.2ml	1	Sc	-	-	-	-	0	0

Variable	Obs	Mean	Std. Err.	[95% Conf. Interval]	
Actual MD	6	5	2.236068	0.7479957	10.748

* Level of protection is based on the expected percentage of MD when using a particular vaccine and challenge virus. An arbitrary value of 25% was considered as a cut-off point: p <25% and np >25% (Gimeno *et al*., 2008)

** Actual percentage of MD indicates the results obtained in this study

- Not applicable

The experimental model used in this study was validated in experiment 1. The result indicated that the mean actual MD incidence 5% was by far lower than the expected cut-off point 25% and it was categorized in p (protected) group. None of the chickens were dead while only Addis Ababa, Dukem and Adama isolates caused tumour lesions in one chicken from their group. This implied that the vaccine was safe and protective against MD in experiment 2.

5. DISCUSSION

In the present study MD outbreak investigation of clinical symptoms like depression, loss of weight, raised and roughened skin around feather follicles, flaccid neck, paralysis of wing and leg were observed. These clinical signs have also been documented as characteristic signs of MD OIE (2010) and Fenner *et al*. (2011). White foci of neoplastic tissue in liver, heart and lung and enlargement of spleen were observed in most of the cases. These post mortem findigs are in conformity with Fenner *et al*. (2011) and OIE (2010). In addition, viruses were isolated from outbreak samples on CEF cell and isolates confirmed to be MDV by using virus specific genotyping methods targeting the ICP4 gene of MD virus (318bp) using conventional PCR. From a total of 46/30 pooled outbreak samples subjected to conventional PCR test, 28 samples (93.3%) were positive for MD while the remaining two samples (6.7%) were found negative. Even though genotyping was not done in the present study, all isolates for central Ethiopia has been clustered under Gallid Herpes virus type 2 (Berhan, 2014) which is MDV serotype 1, known as an oncogenic strain (Shambhu *et al*., 2012). In general, the findings confirmed that the outbreak was caused by MDV.

The questionnaire survey results indicate that chickens at the age of 3-6 months (87.4%) are more affected with MDV than others age group. This finding is in harmony with that of age susceptibility and the disease is most common between 3 and 7 months of age (OIE, 2010). In this study, morbidity in non-vaccinated chickens ranged from 22.2% to 51% and mortality as high as 29%. The recorded mortality is a bit lower than 46% mortality report of Lobago and Woldemekel (2004) in non-vaccinated exotic chickens in central Ethiopia. But, it is in line with the report of Palya (1991) and Blaha (1989) who have indicated high morbidity and mortality that ranges from 20% to70 % of MD in non-vaccinated chickens kept intensively. On the other hand, the morbidity rate in vaccinated flocks ranges from 0.7% to 3.3% and mortality as high as 0.4%. The present morbidity in vaccinated chickens is in complete agreement with Palya (1991) report of less than 5% morbidity vaccinated chickens. Thus, MD vaccination has brought a difference to commercial poultry farms in successfully controlling MD.

The field isolate viruses designated as Addis Ababa, Akaki, Dukem, Bishoftu, Mojo and Adama according to their respective collection site. Serial passage of MDV has been shown to be associated with a reduction of virulence (Churchill *et al*., 1969a; Konobe *et al*., 1979; Witter, 1991a). However, field isolates of MDV pass a limited number of times in CEK and CEF cultures prepared from specific-pathogen-free (SPF) embryos and shown to retain their virulence as indicated in Churchill *et al*. (1969a). In the present study, all isolates were adapted on primary CEF at eighth passage and the virus particle concentration was more than 3000PFU per ml of virus suspension.

The pathogenicity experiment was undertaken to compare the pathogenicity among the six isolates. All chickens challenged by the six isolates (100%) at 1000 PFU dose were died or developed tumour lesions on their internal organs. However, there was no mortality observed in chickens challenged by six isolates at 500 PFU and tumour development was varied from 60% to 80%. Whereas, all negative control birds remained uninfected by both parameters measured. The standard pathogenic isolate cause at least 70% death or lesions in challenged chickens (Anonymous, 1995). Therefore, all six local isolates can be used as standard challenge strains at 1000 PFU. In the absence of standard challenge strain, selection of challenge isolates from the six isolates having equivalent virulence was very difficult. As the result, all six isolates were used as challenge strain to evaluate HVT FC 126 vaccine strain efficacy.

This study revealed that immunized chickens with HVT FC 126 strain and challenged with the local MDV isolates were found to be protected against MDV. None of vaccinated chicken was died during the experiment period irrespective of challenge isolate. Nevertheless, one chicken from each three isolates (Addis Ababa, Dukem and Adama) group was developed tumour lesion. With regards to the six isolates, the protective index of HVT FC 126 strain ranged from 90% to 100% with an average rate of 95% according to Witter (1997) efficacy trail model. This model stated that the protective index should be greater than 75% if the vaccine is to have any protective effect. Vaccination has been known to protect against mortality, but not against the development of lesion in these

groups (Celina *et al*., 2004). However, it is possible that vaccination merely delayed tumour development. Therefore, HVT FC 126 vaccine strain provides a protection to vaccinated chickens against MDV strain circulating in central Ethiopia.

6. CONCLUSION AND RECOMENDATIONS

Marek's disease is one of the major viral diseases of chickens in central part of Ethiopia. It is a highly contagious viral infection that predominantly affects chickens. The high morbidity and mortality of the disease can seriously threaten the poultry industry. The disease mainly affects three to six months of age chickens. Unvaccinated flocks are severely affected by the disease as compared to vaccinated ones although the vaccine couldn't completely stop the morbidity of the disease. HVT FC126 vaccine was effective against six isolates of Marek's disease virus circulating in central Ethiopian chickens. Since these local strains remain pathogenic at eight passages on primary chicken embryo fibroblast cell culture it can be used as a challenge strain in the future. Hence the development and production of effective vaccine for the control of the spread of the virus will enables commercial chicken farms to access the vaccine easily. It has also important implications in the control of Marek's diseases.

Based on the findings of this study the following points are recommended to enhance disease prevention and controlling strategies:

- Further studies should be conducted to be acquainted with the distribution of serotypes and pathotypes of Marek's diseases virus circulating in the country.
- Detailed study should be conducted on field effectiveness of the trial vaccine.
- 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^Oc, 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 37^0C. (7.5ml for $25cm^2$, 25ml for $75cm^2$, and 50ml for $162cm^2$ flasks.
16. Confluency should be obtained within 24 hours.

Annex 2: Chicken Embryo kidney cell (CEKC) preparation

Use 18-19 day old embryos. Kidney cells can also be prepared from day-old or older birds. The amounts indicated here are for preparing kidney cells from 10-15 embryos.

1. Prepare media and trypsin solution and set in 37^{o} C water bath.
2. Spray eggs with Bioguard disinfectant and allowed to dry.
3. Using sterile technique remove embryos with blunt ended curved forceps and put into tray.
4. Either "skin" the embryos (which is the easiest way to get rid of feathers), use regular dissection methods or cut the backbone right above wing joint and separate. This exposes the kidneys without having to touch the intestines and viscera.
5. Remove kidneys and put into glass beaker containing phosphate buffer solution (PBS) without calcium or magnesium (Modified PBS) with antibiotics.
6. Pour off supernatant and clean kidneys. If there are any large chunks, mince lightly with scissors or squeeze gently with forceps. Wash 3-4 times with modified PBS without calcium, magnesium. Use 75-100 ml PBS total.
7. Drain off the last wash and pour the tissue fragments into a trypsinization flask containing a magnetic stir bar. Add 50-100 ml pre warmed (37^{o} C) trypsin-EDTA solution.
8. Put the flask on a stirrer base in 37^{o} C incubator and stir very slowly for 15-20 minutes.
9. When the supernatant is cloudy, shake flask, then set it down for several minutes to let the clumps settle out. Take out 1 drop of supernatant and put it on a glass slide and observe. If there are many single cells and small clumps (2 to 10 cells) with few very large clumps then it is time to pour off the supernatant. Have ready a sterile graduated centrifuge tube with 5 ml of cold heat-inactivated calf serum in it. (Set in a pan of ice.) Pour supernatant through gauze covered funnel into this tube. (The calf serum stops the trypsin action.) With fresh trypsin repeat process 1-2 times (10 min. each) more. Centrifuge at 1500 RPM for 10 minutes.

10. The kidney cells (and RBC's) will pellet. Note the amount of cells obtained. Pour off trypsin solution. Resuspend cells in 3-5 ml of minimal essential medium (MEM) . Add the cells to the appropriate amount of MEM with 10% heat-inactivated fetal calf serum. [One ml of cell pack can be resuspended in approximately 180 ml of MEM]. Cells can be counted in a hemacytometer by resuspending in a known amount of media. Make 1:10 dilution of cells in trypan blue. You will want approximately 2.5 x 10^6cells/ml of media to plate out the cells. The cells should form a monolayer in 1-2 days.

Annex 3: Vaccine production protocol (OIE, 2010; FAO, 1991)

Procedure

Vaccines against MD are prepared from live attenuated strains belonging to the 3 serotypes using CEF as the substrates.

Requirements for substrates and media

Substrate cells are seeded into flat-bottomed vessels for stationary incubation, or into cylindrical vessels for rolled incubation. Media commonly used are Eagle's minimal essential medium, or 199 medium, buffered with sodium bicarbonate and supplemented with 5% calf serum. Incubation is at 38–39°C for 48 hours. For cell-associated vaccine, cultures are infected with production MDV seed-virus stock, in cell-associated form, which is usually two passages beyond the master seed stock. Cultures are incubated for 48 hours then the infected cells are harvested by treating the washed cell sheet with an EDTA/trypsin solution to allow the cells to begin to detach. The flasks are then returned to the incubator (38.5°C) to allow complete detachment. The cells are subjected to low-speed centrifugation, and then resuspended in the freezing mixture consisting of cell growth medium containing 7.5–15% dimethylsulphoxide (DMSO), and held at 4°C or dispensed immediately into the final vaccine containers, usually glass ampoules, which are flame sealed and frozen in liquid nitrogen.

In process controls

For optimal results in preparing cell-associated vaccine, a slow rate of freezing (1–5°C per minute) and rapid thawing are essential. The infectivity titer of the infected cells, and hence the number of doses per ampoule, are determined after filling the ampoules. Similarly for cell-free vaccine, the virus content of the final suspension, and hence the number of doses per container, is determined after filling.

Final product batch tests

Using immunofluorescence assay (IFA) with monospecific serum, checks should be carried out to show that the product is of the same specificity as the seed virus. This is best done using monoclonal antibodies.

Sterility/purity

Extensive testing is required of the materials used to produce the vaccine, and of the final product. Substrate cells should come from an SPF flock, in particular, free from vertically transmitted agents. Substances of animal origin used in the preparation of vaccines such as serum, trypsin, and bovine serum albumin, must be free from extraneous agents. Batches of the final vaccine produced should be tested for freedom from contaminating bacteria, fungi, mycoplasma and the viruses listed for SPF flocks; tests for purity of the diluent should also be conducted. Suitable tests for the detection of extraneous agents at all stages of vaccine production are recommended.

Safety

Ten doses of vaccine or a quantity of diluent equivalent to two doses of vaccine should be inoculated into separate groups of ten 1-day-old SPF chickens. No adverse reactions should occur during a 21- day observation period.

Batch potency

The standard dose of each type of vaccine is 1000 PFU per chicken or egg. Virus content assays are conducted on batches of vaccine to ensure that the correct dose per bird will be achieved

Annex 4: Test for potency - estimation of virus content

Materials and equipment

- VERO cell suspension
- GMEM
- bijoux bottles with Screw caps or silicone rubber stoppers
- Tissue culture plates, 96-well, flat bottom
- Inverted microscope.
- CO2 Incubator

Preliminary steps

Vero cells from one flask are trypsinised and suspended in complete culture medium at 300,000cells/ml.
Place into test tubes or bijoux bottles 4.5 ml of GMEM without serum for preparing the serial dilutions.

Chronological instructions

1. Reconstitute freeze-dried vaccine in 1 ml of cell growth medium. A Liquid viral antigen is used as it is.
2. Set up dilution bottles in rack and mark them in order.
3. Prepare 10-fold dilution series from 10^{-1} to 10^{-6} thus: pipette 0.5 ml of the undiluted liquid vaccine or the reconstituted vaccine into the first dilution bottle, marked 10^{-1} without touching the diluent meniscus. The pipette is discarded. With

a fresh pipette mix the contents of this bottle thoroughly and transfer 0.5 ml to the second bottle, marked $10^{-2,}$ again without touching the meniscus of the diluent. Repeat the mix and transfer sequence until the 10^{-8} dilution, each time with a fresh pipette. This results in a set of 8 bottles containing serial dilutions of the vaccine from 10^{-1} through 10^{-8}. (Separate sterile pipettes must be used for each dilution of the vaccine. After the required volume of mixture is transferred to the next tube, change the pipette).

4. Dispense 0.1 ml culture medium into each of 6 first wells of column 12.
5. Dispense 0.1ml of vaccine dilution 10^{-6} into each of 10 wells of column 1 to 10 in Row F and continue for every dilution step towards Row A (dilution 10^{-1}).
6. Add 0.1ml of Vero cell suspension (30,000 cells per well, equivalent to 100 µl of cell suspension) to each of the six first well in column 12.
7. Add 0.1 ml of the cell suspension (30,000 cells per well, equivalent to 100 µl of cell suspension). to each row of column 1 to 10 starting from row F to row A.
8. Column 11 should therefore be empty.
9. One row of wells serves as a control for uninfected cells to which
10. Add 0.1ml of GMEM into each of the first 6 wells of column 12 (control of uninfected cells).
11. Seal and incubate plates in 5% CO_2 atmosphere at 37^oC for 10-15 days.
12. Using an inverted microscope, fitted with a specimen guide microplate template and 2.5x and 10x objectives, examine the monolayers for evidence of CPE, initially, on day 4-5 and, thereafter, on every second day until day 10-11.
13. Record wells in which the cells are showing CPE. Cells in column 12 should be compact and devoid of any evidence of CPE.
14. Calculate the virus titre using the Spearman-Karber formula (Titre/ml, titre/dose).

Calculations and Interpretations of the result(s)

The calculation of virus titer relies on the principle of quantal dose response relationship. For a stimulus - subject system as virus titration, measurement of response is to record whether or not the subject manifests the expected reaction. The quantal assay so used

measures an "all - or - none" response, e.g. CPE as a manifestation of infection. To measure such a quantal response, the most frequently used system is the multiple serial dilution assays. In a multiple serial dilution assay, each dilution is tested in replicates (at least five). The end-point is the dilution of a substance at which a specified number of members of a test group shows a defined effect. The most frequently used and statistically useful end-point is 50%. It is the ***Median Effective Dose***, which in virus infectivity titration in cell cultures is the *Tissue Culture Infective Dose (*$TCID_{50}$*)*. Thus the median effective dose is the dilution of the test population which will demonstrate response in 50% of the population. i.e CPE in 50% of a large number of inoculated cultures.

Procedure using the Spearman-Karber Formula:

The test sample is diluted in a geometric series, that is, with a constant ratio between successive dilutions, and a constant volume (usually 0.1ml) of each dilution is inoculated into each of at least five replicate cell cultures. The most commonly used dilution factor is 10-fold.

For the Spearman-Karber formula to be applicable it is necessary to use constant number of test monolayers per dilution (n_i), a constant dilution factor and a range of dilutions wide enough to bracket both the dilutions at and below which 100% of n_i subjects (i.e. cell culture monolayers) tested will respond and the dilutions at and above which 100% of n_i subjects test will be negative.

If one or more of these conditions is not met, it is sometimes assumed that, for a constant dilution factor, the next higher or lower dilution to the last one tested would have produced the desired result. The "fabrication" of data in this way is without any theoretical basis, but if applied with suitable caution it may do little harm. However, it is preferable to repeat the titration with more appropriate range of dilutions, and this is essential if there are serious shortcomings in the data.

The Spear-man Karber formula:

Log10 Median Dose = $(X_o - (d/2) + d*(\sum r_i/n_i)$ where

- X_o $\log^{10}$ of the reciprocal of the lowest dilution at which all test monolayer are positive.
- d $\log^{10}$ of the dilution factor (i.e. the difference between the log dilution intervals)
- n_i number of test monolayer used at each individual dilution (after discounting accidental losses)
- r_i number of positive test monolayers (out of n_i).
- $\sum(r_i/n_i)$ sum(P), sum of the proportion of positive tests beginning at the lowest dilution showing 100% positive result.The summation is started at dilution X_o .

NB: When using the Spearman-Karber method, it has to be borne in mind that random variation in the number of positive monolayers will cause small but unknown deviations from the true values of the end-point dilutions. These deviations will be large if only a small number of monolayers per dilution is used. There may also be slight inaccuracies resulting from the method of estimation itself, but it has been shown that, on the whole such inaccuracies are smaller with the Spearman-Karber method than with other comparable methods (e.g. using the Reed-Muench formula).

If the value of n_i (number of test monolayers used at each individual dilution) has been reduced by accidental losses (e.g contamination, degeneration etc.) it is still possible to obtain a valid estimate of the median effective dose using the Spearman-Karber formula.

Estimated Standard Error

The estimated Standard Error is calculated using the following formula:

Log St Error = d*sq rt[sum $(p*(1-p)/(n_i-1)]$

Titer per dose

For Marek's disease vaccine, the minimum titer per field dose, calculated on the basis of the mean of at least 3 separate titrations, should be at least 10^3 PFU. However, it is recommended in order to safeguard against poor storage condition to deliver vaccine with a titer of $10^{3.2}$ PFU.

Annex 5: Growth and maintenance media

Growth medium consist of GMEM supplemented with 10% tryptosephosphate 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 and CEKC, this medium will be replaced after 24 h by the same medium but supplement with only 2-5% FBS.

Annex 6: DNA extraction from tissue sample (Qiagen, Germany)

- Cut tissue (≤ 10mg spleen or ≤ 25mg other tissue) into small pieces, and place in a 1.5 ml microcentrifuge tube. Add 180 µl Buffer ATL. Add 20µl proteinase K, mix by vortexing, and incubate at 56°C until completely lysed. Vortex occasionally during incubation. Vortex 15 s directly before proceeding to step 2.
- Add 200 µl Buffer AL. Mix thoroughly by vortexing. Incubate samples at 56°C for 10 min.
- Add 200µl ethanol (96-100%). Mix thoroughly by vortexing.
- Pipet the mixture into a DNeasy Mini spin column placed in a 2 ml collection tube. Centrifuge at ≥ 6000 x g (8000 rpm) for 1 min. Discard the flow-through and collection tube.
- Place the spin column in a new 2 ml collection tube. Add 500 µl Buffer AW1. Centrifuge for 1 min at ≥6000 x g. Discard the flow-through and collection tube.

- Place the spin column in a new 2 ml collection tube. Add 500 µl Buffer AW2, and centrifuge for 3 min at 20,000 x g (14,000 rpm). Discard the flow-through and collection tube.
- Transfer the spin column to a new 1.5 ml or 2 ml microcentrifuge tube.
- Elute the DNA by adding 50µl Buffer AE to the center of the spin column membrane. Incubate for 1 min at room temperature (15-25^{o}C). Centrifuge for 1 min at ≥ 6000 x g.

Annex 7: Agarose Gel Electrophoresis of PCR product

1. Prepare 60 ml of 1.5 % agarose in 1x TBE buffer.
2. Either heat in microwave for ~2 min on full powder or place in beaker of being of boiling water until melted.
3. Allow to cool to about 450C and add 2.5 µl /ml red gel.
4. Pour gel and insert well former (comb). Allow to set on a flat surface for about 15 min.
5. Pour buffer 1x TBE into tank and remove comb from gel.
6. Prepare samples in tubes, a multiwell plate or on parafilm.
   1 µl loading buffer
   5 µl PCR product
7. Prepare molecular weight marker.
   0.5 µl ml molecular weight marker VI (Boehringer)
   1 µl loading buffer
   4.5 µlH_2O
8. Load samples into the wells formed in the gel. It is often useful to load the molecular weight markers in both the first and last lanes.
9. Electrophoreses at 100 volts for 20 min (minimum) or 10 volts overnight.
10. View and photograph the gel on UV- transilluminator. Use UV- safety spectacles.

Annex 8: DNA extraction

DNA extraction from cultured cells sample:

- Centrifuge a maximum of 5 x 106 cells for 5 min at 300 x g (190 rpm). Re-suspend in 200 µl PBS. Add 20µl proteinase K. Proceed to step 2.
- Add 200 µl Buffer AL. Mix thoroughly by vortex. Incubate blood samples at 56^{o}C for 10 min.
- Add 200µl ethanol (96-100%). Mix thoroughly by vortex.
- Pipet the mixture into a DNeasy Mini spin column placed in a 2 ml collection tube. Centrifuge at ≥ 6000 x g (8000 rpm) for 1 min. Discard the flow-through and collection tube.
- Place the spin column in a new 2 ml collection tube. Add 500 µl Buffer AW1. Centrifuge for 1 min at ≥6000 x g. Discard the flow-through and collection tube.
- Place the spin column in a new 2 ml collection tube. Add 500 µl Buffer AW2, and centrifuge for 3 min at 20,000 x g (14,000 rpm). Discard the flow-through and collection tube.
- Transfer the spin column to a new 1.5 ml or 2 ml microcentrifuge tube.
- Elute the DNA by adding 200µl 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.

Annex 9: Phosphate buffered saline (PBS)

Ingredient	Amount
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 10: Trypsin Solution (0.25%)

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

Adjust pH to 7.4 with NaHCO3 solution

Annex 11: SPGA/ EDTA Buffer Preparation

(Sucrose, phosphate, glutamate and albumin/ethylenediamine tetra-acetic acid)

Ingredient	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

Adjust pH at 6.5

Annex 12: Marek's Disease field outbreak assessment questionnaires form

Region: _____

Zone: _____

Type of disease suspected: _____

Date: _____

Farm history

Production type: _____

Breed _____

Sex _____

Age _____

Total population _____

No. Sick _____

No. death _____

Vaccination history _____

Clinical sign observed

_____

_____

_____

_____

Post mortem finding

_____

_____

_____

I. Analyzed table of Questionnaire

No.	Code	Breed	Sex	Age	Production type	No. Sick	No. at risk	No. death	Vaccination history	Sample type	Address
1	VR 721/15	Bovans Brown	Female	5.5 month	layers	15	435	2	Vaccinated (HVT Fc126)	Spleen, liver lung	Addis Ababa (kolfe)
2	VR 720/15	Bovans Brown	Female	5 month	layers	1500	2400	594	Unvaccinated	Spleen Feather	Bishoftu
3	VR 711/15	Bovans Brown	Female	1 year	layers	5	500	1	Vaccinated (HVT Fc126)	spleen	Dukem
4	VR 692/15	Bovans Brown	Female	6 month	layers	104	40	60	UN	spleen	Akaki
5	VR 485/14	Bovans Brown	Female	5 month	layers	800	2400	400	Unvaccinated	spleen	Dukem
6	VR 409/14	Bovans Brown	Male	20 days	broilers	20	1800	5	Vaccinated (HVT Fc126)	spleen	Bishoftu
7	MB 768/15	Bovans White	Female	5 month	layers	30	1500	0	Vaccinated (HVT Fc126)	Liver, spleen feather	Mojo
8	MB 738/15	Bovans Brown	Female	3 month	layers	40	6000	10	Vaccinated (HVT Fc126)	Spleen feather	Adama