PREVALENCE OF MAREK'S DISEASE AND DETECTION OF MAREK'S DISEASE VIRUS STRAINS USING TWO MOLECULAR TECHNIQUES IN JOS, PLATEAU STATE, NIGERIA

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BY

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OCTOBER, 2015

DECLARATION

I declare that the work in this dissertation entitled "**Prevalence of Marek's Disease and Detection of Marek's Disease Virus Strains Using Two Molecular Techniques in Jos, Plateau State, Nigeria"** has been carryout by me in the Department of Veterinary Medicine. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of the dissertation was previously presented for another degree or diploma at this or any other Institution.

Adeyinka Jeremy ADEDEJI _____ _____

Name of Student **Signature** Date

CERTIFICATION

This Dissertation entitled "**PREVALENCE OF MAREK'S DISEASE AND DETECTION OF MAREK'S DISEASE VIRUS STRAINS USING TWO MOLECULAR TECHNIQUES IN JOS, PLATEAU STATE, NIGERIA"** by Adeyinka Jeremy ADEDEJI meets the regulations governing the award of the degree of M.Sc. Avian Medicine of the Ahmadu Bello University Zaria and is approved for it contribution to knowledge and literary presentation.

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DEDICATION

This work is dedicated to God ALMIGHTY to whom I am eternally grateful.

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ABSTRACT

Marek's disease (MD) is a neoplastic and immunosuppressive disease of poultry. Marek's disease have been reported in several parts of Nigeria, however information on the actual prevalence of the MD is lacking. The aim of the study was to determine the prevalence of MD and detect the different strains ofMD virus using polymerase chain reaction (PCR) and loop mediated isothermal amplification procedure (LAMP). The prevalence of MD in Jos Plateau State for year 2007-2013 was determined through analysis of records from five selected veterinary clinics and one national veterinary diagnostic centre. Factors such as year, month, age, season, species and concurrent infection were used for analysis of the results. The detection of different serotypes and strains of MD virus in Jos Plateau State was carried out usingPCR and LAMP onsamples collected from clinically diagnosed cases of MD. Samples collected were spleen, feathers, liver, skin, heart, kidneys and lungs from different types and species of poultry during post-mortem examination. The overall prevalence of MD in Jos, Plateau State was 1.99% between the years 2007-2013. Analysis of results showed that MD was diagnosed in broilers (4.73%), layers (46.23%) and turkeys (0.72%) retrospectively. The records also showed that MD was concurrently diagnosed with coccidiosis (6.65%), colibacillosis (4.20%), salmonellosis (0.18%), Newcastle disease (8.41%), Fatty liver syndrome (0.18%) and Fowl typhoid (1.05%).It was concluded that, the detection rates of different serotypes and strains of MDV in Jos, Plateau State using PCR, wereof Marek's disease virus (MDV) Serotype 1 (RB-1B) (87.90%), MDV Rispens strain (84.40%), MDV pathogenic strain (Australian strain) (7.94%) and MDV (HVT) serotype 3 (FC 126) (9.09%). In addition, MDV RB-1B strain was detected in feathers (90.38%), liver (100%), spleen (81.33%), heart (100 %), lungs (100%), kidneys (100%) and skin (100%). While MDV Rispens strain was detected in feathers (89.36%), liver

(100%), spleen (78.95%) and skin (100%). The MDV pathogenic strain was detected in feathers (5.88%) and spleen (11.54%) while HVT serotype 3 (FC 126) was also detected in feathers (3.13%), liver (100%) and spleen (14.29%).The MDV RB-1B strain was detected in broilers (88.0%), layers (84.88%), local chickens (75%) and quails (100%), while MDV Rispens strain was detected in broilers (86.67%), layers (83.52%), local chickens (50%) and quails (100%). Furthermore, MDV pathogenic strain was detected only in layers (12.5%) and HVT serotype 3 was detected in broilers (16.67%), layers (12.50%) and local chickens (33.33%).Samples positive by PCR (85.7%) for MDV RB-1B were also diagnosed to be MD by gross examination and histopathology. Loop mediated isothermal procedure was used to detect HVT FC 126 in liver (100% 1/1), feathers (16.6%, 1/6) and skin (100% 1/1). In addition, MDV HVT FC 126 was detected in broilers, layers and local chickens. It was therefore, recommended that more work should be carried out by Research Institutes and Veterinary schools to fully understand the epidemiology and diagnosis of the MD in Jos and other parts of Nigeria. Field veterinarians should improve their record keeping of clinical cases and criteria of differentiating MD from other neoplastic diseases of poultry, so that the actual prevalence of MD in Jos, Plateau State will be known.Researchers in Research Institutes and Universities need to understand and optimize the use of LAMP which is cheaper, faster and more sensitive than PCR so that it can be used by small laboratories for confirmatorydiagnosis ofMD in Nigeria.

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LIST OF ABBREVIATIONS

ADCC	Antibody- dependent cell dependent cytotoxicity
AGPT	Agar gel precipitation test
ALV	Avian leucosis virus
BIP	Backward inner primer
CEF	Chicken embryo fibroblast
CK	Chicken kidney cells
CVI988	Strain of Serotype 1 Marek's disease virus
DEF	Duck embryo fibroblast
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraactic acid
ELISA	Enzyme-linked immunosorbent assay
FFE	Feather follicle epithelium
FIP	Forward inner primer
HVT	Herpes virus of turkeys
HVT FC126	A strain of Marek's disease virus serotype 3
IFN-c	Interferon-gamma IgM Immunoglobulin M
IgG	Immunoglobulin G
NASBA	Nucleic acid sequence-based amplification
LAMP	Loop mediated isothermal amplification Procedure
MATSA	Tumour-associated antigen
MD	Marek's disease
MDV	Marek's disease virus
MHC	Major Histocompatibility Complex
ml	mililitre
mMDV	mild Marek's disease virus
ND	Newcastle disease
NK	Natural killer cells
NO	Nitric Oxide
PCR	Polymerase Chain Reaction
qPCR	Quantitative real time Polymerase Chain Reaction

RNA	Ribonucleic acid
RT-PCR	Reverse-transcriptase Polymerase Chain reaction
SB1	Strain of serotype 2 Marek's disease virus
ED	Embryonated day
RB-1B	A strain of serotype 1 Marek's disease virus
REV	Reticuloendothelosis virus
SARS	Severe acute respiratory syndrome
µl	microlitre
vMDV	Virulent Marek's disease virus
vvMDV	very Virulent Marek's disease virus

CHAPTER ONE

INTRODUCTION

1.1 Background

Marek's disease (MD), named after the Hungarian pathologist József Marek, is a lymphoid neoplasia of domestic chickens and turkeys, quails and geese (Biggs and Nair, 2012). Originally described in 1907 as a polyneuritis affecting the peripheral nerves, it was not until 1926 that MD was recognized as a neoplastic disease associated with tumours in several visceral organs (Nair, 2008). In Nigeria, MD was first reported in 1962,since then there have been reports in Ibadan,Jos and other parts of the country(Hill, 1962; Nawathe*et al.,* 1978;Adene and Akpavie, 1997; Olabode *et al.,* 2009; Okwor and Eze, 2011; Wakawa *et al.,* 2012).

Marek's disease is caused by MD virus (MDV),which is a double-stranded linear DNA virus belonging to the family*Herpesviridae*, subfamily *Alphaherpesvirinae*, genus *Mardivirus*, specie*Gallid Herpesvirus* 2 (GaHV-2)(Renz *et al.*,2006). Marek's disease virus strains are classified into 3 serotypes, GaHV-2 (MDV serotype 1: MDV-1), *Gallidherpesvirus* 3 (MDV serotype 2: MDV-2) and *Meleagrid herpesvirus* 1 (MDV serotype 3 or Herpesvirus of turkeys (HVT). Marek's disease is caused by serotype1 MDV strains except for attenuated vaccine strains (Matsuyama-Katoat*et al.,* 2012).Serotype 1, which includes all the pathogenic or oncogenic strains of MDVis further classified into the following pathotypes: mild MDV, virulent MDV, very virulent MDV, very virulent + MDV (Payne,2000).

Chickens are the most important natural host for MD, but quails, turkeys, and pheasants,game fowl and jungle fowl are susceptible to MDV infection and tumor development (Witter and Schat, 2003). MDV serotype 1 has been demonstrated in migratory and sedentary waterfowl (Murata *et al.*, 2011).

Although MD incidence has become low in some parts of the world, it remains a significant problem in many countries, especially in areas of French-speaking Africa, Eastern Europe, East Asia, and South America (Dunn and Gimeno, 2013). In commercial chicken houses, where the infection is rampant, virtually all the birds become infected, commonly within the few weeks of life, although on some occasions, infection may be delayed. The unique epidemiological features of MD, such as the highly contagious nature, the widespread distribution and the long-term infectivity of the poultry house environment, make the eradication of the disease almost impossible (Marrow and Fehler, 2004). Prevention of the disease is essentially based on vaccination, although improved biosecurity and genetic resistance can contribute to the control (Marrow and Fehler, 2004). The development of MD vaccines was a significant landmark both in avian medicine and basic cancer research, as this was the first example of a neoplastic disease controlled by the widespread use of a vaccine (Gimeno, 2004).

In affected flocks, birds appear unthrifty, have delayed feathering, have ruffled feathers, and are stunted, in addition other non-specific signs may be seen which include weight loss, paleness, anorexia, and diarrhoea, especially in birds in which the course is prolonged (Witter and Schat, 2003). Specific signs include paralysis of the legs and wings, blindness and emaciation (Witter and Schat, 2003). Under commercial conditions, death often results from starvation and dehydration because of the birds'

inability to reach food and water or, in many cases, from trampling by flock mates (Witter and Schat, 2003).

Diagnosis of MD is based on history, clinical signs, post-mortem findings, microscopic lesions and the laboratory diagnosis.There have been several advances in the laboratory diagnosis of MD, the most commonly used methods are the use of virus isolation, agar gel immunodiffusion test, enzyme-linked immunoasorbent assays (ELISA) for detecting MDV antibodies (Zelnik, 2004).Molecular techniques have been developed for the detection and quantification of MDV field and vaccine strains (Cortes *et al.,* 2009). Currently, detection of MD in different samples, including feather tipscan be done by PCR or real-time PCR-based (Cortes *et al.,* 2009). These techniques have provided remarkably important tools in the diagnosis and characterization of MDV infection.

However, the requirement of using thermocyclers still presents a problem for routine testing by small laboratories and field veterinarians(Woźniakowski *et al.,* 2011). Therefore, despite the availability of different diagnostic tests, there is still need for a rapid, inexpensive, and simple molecular method that does not require expensive equipment (e.g. plate readers, thermocyclers, gel apparati). Alternative methods to standard PCR include nucleic acid sequence-based amplification (NASBA), ramification amplification, and loop-mediated isothermal amplification procedure (LAMP) (Woźniakowski *et al.,* 2011).

Loop-mediated isothermal amplification procedureis based on amplification of specific genetic loci using isothermal conditions provided by a water bath or heating block (68–70 C) and Bst or Bsm polymerases, which have DNA strand displacement activity (Notomi *et al.,* 2000; Woźniakowski *et al.,* 2011). The technique can amplify a few

copies of DNA to 10^9 in less than one hour under isothemal conditions (Wei *et al*., 2012). LAMP is a rapid, sensitive innovative technique developed and evaluated for the detection of MD virus (MDV) by amplification of conserved MDV meq gene sequences (Xiuying *et al.,* 2012).

1.2Statement of Research Problem

There is an increasing growth of backyard and commercial poultry farms in Plateau State and Marek's disease is increasingly becoming a viral disease of economic importance in Nigeria (Wakawa *et al.*,2012). Acute MD usually manifest clinically when birds have reached the grower stage which is 11-20 weeks of age with mortality rate up to 60% (Marrow and Fehler, 2004; Okwor and Eze, 2011; Wakawa *et al.*, 2012).

In a survey conducted in 2004, Nigeria is one of the 55 countries around the world that have experienced economic losses from MD since the 1990s (Gimeno, 2004). Since the first report of MD in Nigeria by Hills in 1962, there have been several reports of outbreaks of MD(Nawathe *et al.*,1978;Fatunmbi and Adene, 1986; Owoade *et al.*, 2008; Olabode *et al.*, 2009;Okwor and Eze, 2011; Jwander *et al.*, 2012; Wakawa *et al.*,2012). In a recent report in 2013, Nigeria is one the countries with increasing prevalence of MD in the past 10 years, despite being one of the countries that uses Rispens CV1988 vaccines for vaccination and revaccination of birds against MD (Dunn and Gimeno,2013).

Marek's disease has been successfully controlled by vaccination since 1969 but despite the great efficacy of MD vaccines, however, it has a dark side; the use of MD vaccines has been accompanied with the increase in virulence of MDV(Gimeno, 2008).

Vaccination against MDV does not lead to a sterilizing immunity. This means the birds are protected from clinical disease but not from infection, and shedding of MDV from the feather follicles can still occur (Marrow and Fehler, 2004; Dunn *et al.,* 2010).

In Nigeria, the fact that MD vaccines are not locally produced has resulted in the introduction of MD vaccines from different countries into the country. This may presents risks for MD infection among poultry flocks in the country and also evolution of MD variants which may be considered as the likely causes of MD outbreaks in vaccinated flocks (Davison and Kaiser, 2004; Murata *et al.*,2012; Wakawa, *et al.,* 2012).

1.3 Justification of the Study

Poultry farming is a common source of livelihood in Jos, Plateau State and the present status of MD is unknown, the last reported work in Jos, Plateau state was done in1978 ((Nawathe *et al.,* 1978).Although work was done on MD in 2005covering part of Plateau state, the emphasis was on the molecular detection of the disease(Jwander *et al*., 2012). Marek's disease causes severe immunosuppression that makes its host vulnerable to tumour development as well as to other pathogens which may further increase the cost of managing the birds (Davison and Kaiser, 2004; Schat, 2004; Gimeno, 2013). In addition, MD results in severe economic loses to the poultry industry and pose constant threat to poultry farms. The ability to detect MDV during the early stages of an outbreak is an important step in the containment process (Xiuying *et al.,* 2012).

Confirmatory diagnosis of MD is usually a problem because of lack of laboratory back up for veterinarians in the field.In addition there is a need for accurate and prompt

diagnosis of MD because of the nature of the disease which manifest at the grower stage (11-20 weeks) of age with mortality rate of up to 60%(Marrow *et al.*,2004; Okwor *et al.*,2011; Wakawa *et al.*,2012). Prompt diagnosis will help the farmers to make the right decisionon whether to maintainthe flock or cull them. Accurate diagnosis will also help to confirm whether outbreaks are due to vaccine breaks or failure as it has been reportedthat incidence of increased virulence of MDV has been concomitant with vaccination against MD (Gimeno, 2008).Moreover there is usually similarity in the clinical and gross pathological presentation ofAvian leukosis J,Reticuloendotheliosis and MD, hence the need to differentiate the three viral induced oncogenic diseases(Witter and Schat, 2003). This work would provide easy method for diagnosis of MD and enumerate some epidemiologic factors affecting the prevalence of the disease.

1.4Aim of the Study

The aim of the study was to determine the prevalence of MD and detect MD virus strains using two molecular techniques in Jos, Plateau State

1.5Objectives of the Study

The objectives of the study were to:

1. carryout a retrospective study of MD in Jos, Plateau State.
2. determine the concurrent occurrence of MD with other diseases of poultry in Jos, Plateau State.
3. application of PCR and LAMP as diagnostic techniques for detection of different serotypes and strains of MD virus in Jos, Plateau State.

1.6 Research Questions

1. What is the prevalence of MD in Jos, Plateau State?
2. Does MD occur concurrently with other diseases of poultry in Jos, Plateau State?
3. What are the different serotypes and strains of MD virus circulating in poultry farms in Jos, Plateau State?
4. Can LAMP be used to detect MDV in Jos, Plateau State?

CHAPTER TWO

LITERATURE REVIEW

2.1 History of Marek's Disease

Marek's disease, named after the Hungarian veterinary pathologist over 100 years ago, is a major disease affecting poultry health worldwide (Biggs and Nair, 2012). Marek's disease was first described by Jozsef Marek in 1907 as fowl paralysis (polyneuritis), was later shown to be associated with lymphoid tumours (Pastoret, 2004). The clinical disease has similarities to avian leukosis, and it was about 40 years after that the two conditions were differentiated and the causative agent, Marek's disease virus (MDV) was identified. The first, on clinical diagnosis, was published in Budapest in 1902. The three volumes of *Veterinary Internal Medicine*, edited together with Ferenc Hutya, were published in 1904, and a second edition in two volumes in 1923/1924. This work helped to lay the foundations for the discipline of veterinary internal medicine based on scientific methodology (Payne, 1985; Pastoret, 2004). The importance of this work is shown by the fact that it was translated into English, Italian, Russian, Spanish, Turkish, Serbian, Polish and Chinese (Pastoret, 2004).

Marek's disease (MD) came into being as the name for a disease of poultry in 1960, at the First World Veterinary Poultry Association Conference in Utrecht after presentation by Campbell and Biggs in order to separate the disease from lymphoid leukosis because pathological names been used as at that time led to confusion in the understanding of the disease (Biggs, 2004). After seminal report by József Marek, published in 1907 of paresis in 4 roosters which is the first account of the disease, outbreaks were reported as early as 1914 in the United States. Subsequently, the disease was recognized in the Netherlands, Great Britain, Germany 1927, South Africa 1928, England 1929, Japan

1930, and many other countries (Payne, 1985; Witter and Schat, 2003). The detailed description by Pappenheimer and colleagues clarified the lymphoproliferative nature of the lesions in peripheral nerves and spinal ganglia and established, as part of the syndrome, lymphoid neoplasms in the ovary and other visceral organs. Other early descriptions of MD were by Kaup, 1921; van der Walle and Winkler-Junius, 1924 suggested that it was a condition affecting only the nervous system, which was variously described as polyneuritis, paralysis of the domestic fowl, and neuromyelitisgallinarum (Pastoret, 2004). Kaup described clinical features of the disease as perivascular round cell infiltration in the spinal cord and regarded the disease as transverse myelitis, while Van der walle and Winkler-Junius observed fowl paralysis in the Netherlands (Payne, 1985). In order to understand the disease researchers between 1933-1939 in many countries tried to experimentally transmit fowl paralysis to better understand it cause and pathogenesis, the report of the investigation were given in great details but none gave conclusive evidence of transmission of the disease although the reports were highly suggestive (Payne, 1985). For the next two decades (1940-1959), many workers were working to unravel the mystery of the disease without much success and MD was becoming a much more serious disease, inflicting a severe economic loss to the poultry industry. Mortality rates were between 10 to 30% and on occasion they could reach 60 to 80% in countries with a well-developed or developing poultry industry (Payne, 1985; Biggs and Nair,2012).

One of the major steps forward in the history of MD occurred some six decades after the first description of the disease by Marek, with the isolation of the causative herpesvirus, the MDV, at the Houghton Poultry Research Station, Houghton, Huntingdon, Cambridgeshire. Propagation of the causative agent in cell culture by Churchill and

Biggs in 1968 and independently in the USA by Nazerian, Solomon and their fellow researchers in1968, was a major breakthrough in MD research, and this was soon followed by transmission of the disease with the cell culture-propagated virus (Witter and Schat, 2003; Pastoret, 2004). Subsequently within two years of the discovery of the causative agent of MD, which was identified as a herpesvirus, the virulent isolate HPRS-16 was attenuated by Churchill and his colleagues at Houghton and used as an effective vaccine. Thus MD became the first and most important disease model in which a neoplastic condition was successfully controlled by vaccination (Pastoret, 2004). Shortly after this in 1972, Rispens and his colleagues in the Netherlands described the use of the attenuated MDV strain CVI988 (also known as the Rispens strain), a highly immunogenic strain that is used widely today as the most effective vaccine against MD (Payne, 1985; Witter, 1985; Pastoret, 2004).

The MD virus is cell-associated in all infected cells of an infected chicken except the feather follicles, where cell free virus could be isolated. This provided an explanation for its contagious nature and the infectivity of poultry dust, which is partly made up of chicken dander (Biggs and Nair,2012). In proceeding years, 1972-80 there were a lot of research on the vaccines and vaccination of MD, with reports of differences in the efficacy of the cell free herpes virus of turkeys (HVT) and cell associated HVT vaccines and the vaccinal immunity which shed more light on how the vaccination against MD works (Biggs and Nair,2012).

In Nigeria, MD was first reported in 1962 by Hills in Ibadan,since then there have been reports by Adene in Ibadan (1975) and Nawathe in Jos in 1978 (Adene *et al*., 1997). There were also reports of outbreaks of MD between 1975-1984 in South West Nigeria

with explosive morbidity and mortality during this period; there were two noticeable peaks of outbreaks in 1977 and 1983 (Fatunmbi *et al.,* 1986). Other parts Nigeria have also reported of outbreaks of MD, in Ilorin, Nsukka and Zaria leaning credence to the fact that MD is becoming a common disease of poultry in Nigeria (Olabode *et al.*, 2009; Okwor *et al.,* 2011; Wakawa *et al.,* 2012)

2.2 Aetiology of Marek's Disease

Marek's disease (MD) is one of the most economically devastating infectious diseases of poultry, caused by the highly infectious, cell associated MD virus (MDV), (Hassanin *et al.,* 2013). MDV is one of the most potent oncogenic herpesvirus known (Nair *et al.,* 2003).

2.2.1 Classification of Marek's disease virus

According to the International Committee on Taxonomy of Viruses recent classification of herpesvirus, MDV belongs to the Order *Herpesvirales,* Family *Herpesviridae*, Subfamily *Alphaherpesvirinae* and Genus *Mardivirus* (Davison, 2010; Hassanin *et al.,* 2013). There are three members of the genus *Mardivirus,* Marek's disease herpesvirus serotypes 1 and 2 (MDV-1 and MDV-2) as well as serotype 3 or herpesvirus of turkeys (HVT) (Davison *et al.,* 2002; Nair, 2005). The *Mardivirus* genus was established because of accumulated genomic information clearly indicated their affiliation with the *Alphaherpesvirinae* subfamily of the *Herpesviridae,* but distinguished them from the other genera of the subfamily, such as the *Simplex-*, *Varicello-* and *Laryngoviruses* (Osterrieder *et al.,* 2004).Newer nomenclature classify MDV-1 as gallid herpesvirus II, MDV-2 as gallid herpesvirus III, and HVT as meleagrid herpes virus 1(Osterrieder *et al.,* 2006; Hughes *et al* 2007;Mahy, 2009; Davison, 2010; Hassanin *et al.,* 2013).

2.2.2 Pathotypes of Marek's disease virus

All virulent or oncogenic strains of MDV belong to serotype 1, and currently four pathotypes are recognized as follows:

(a) Mild MDV(mMDV) - in genetically susceptible chickens these strains cause mainly neural MD and sometimes a low incidence of mainly ovarian lymphomas; their pathogenic effects are preventable with HVT vaccines(Payne, 2004).

(b) Virulent MDV)(vMDV) - these cause a high incidence of visceral and neural lymphomas; their effects are prevented by HVT vaccines(Payne, 2004).

(c) Very virulent MDV(vvMDV) - these cause a high incidence of visceral and neural lymphomas. They are oncogenic in HVT vaccinated birds and in birds genetically resistant to less virulent viruses; their effects are preventable with bivalent vaccines(Payne, 2004).

(d) Very virulent plus MDV (vv + MDV) - these cause a high incidence of lymphomas and are oncogenic in birds vaccinated with bivalent vaccines (Payne, 2004).

2.2.3 Viral properties of Marek's disease virus

Marek's disease virus is a DNA virus, which is linear double stranded DNA virus which range in size of 108-230 kbp (Kato *et al.,* 1985; Witter and Schat, 2003; Osterrieder *et al.,* 2004; Osterrieder *et al.,* 2006).

A total of six different general genome organizations has been identified for *Herpesviridae* , referred to as classes A through F. Only class D and E genomes are found in the *Alphaherpesvirinae,* and MDV-1, MDV-2 and HVT represent class E genomes, an organization that is identical to that of the prototype representative of the virus subfamily, herpes simplex virus type 1(HSV -1) (Osterrieder and Vautherot , 2004). The sequence similarity of virus proteins between the three viruses ranges from

50% to 80%, but despite the similarity the viruses vary considerably with regard to their genome sequences (Osterrieder and Vautherot , 2004; Osterrieder *et al.*, 2006). The viruses differ with regards to guanine and cytosine (GC) content and size, whereas the GC content of MDV-1 is as low as 44.1 per cent, that of MDV-2 is 53.6 per cent and interestingly, HVT has a GC content that lies in between these two extremes (47.2 per cent), a total of 99 (HVT), 102 (MDV-2) and 103 (MDV-1) genes have been clearly identified respectively for each of the viruses (Osterrieder and Vautherot, 2004). The rather large variation in basecomposition and genome size, as well as the relatively low homology of individual proteins that reaches a maximum identity of 88 per cent between any of the three viruses, clearly suggests an independent but probably parallel evolution of separate and different virus species(Osterrieder and Vautherot, 2004).Like in all alphaherpesviruses, the genomes of MDV, GaHV-3 and HVT consist of a unique long (U_L) and a unique short (U_S)segment bracketed by inverted repeats known as terminal and internal repeats long (TR_L and IR_L) and terminal and internal repeats short (TR_S and IR_S), respectively (Osterrieder *et al.*, 2006; Nair, 2013).

Marek's disease virus serotype-1 is the only virus of the three that causes clinical disease in chicken, while the other two species are non-pathogenic which may be attributed to Meq and pp 38 genes which are found in this virus only and are implicated for tumour formation in susceptible host (Osterrieder and Vautherot, 2004; Osterrieder *et al.*, 2006; Nair, 2013).

Marek's disease virus is ether sensitive, the nuclear capsid is 90-100nm in diameter and contains 162 capsomeres arranged in icosahedral symmetry (Wakenell *et al.*, 2008). Cell-free MDV obtained from extracts of skin or feather pulp retains its titer for several

months at -60 ^{0}C but not at -20^{0}C. The Virus is inactivated in 2 weeks at 4^{0}C, in 4 days at 22-25^{0}C, 18 hours at 37^{0}C, in 30 minutes at 56^{0}C and in 10 minutes at 60^{0}C. The virus can withstandat least four freeze-thaw cycles and 10 minutes of ultrasonic vibration. MDV is inactivated at PH lower than 4 or higher than 10 (Wakenell *et al.*,2008).

The MDV-1 has the following strains Md5, GA, CU-2, Md11 (BAC), CVI988 (BAC), RB-1B [BAC], 584A [BAC], while the known strains of MDV-2 is HPRS24 and HVT is FC126 (Davison, 2010).

Pathogenic MDV-1 strains have been generally classified virulent, (vMDV); very virulent, (vvMDV) very virulent plus MDV (vv+MDV) with the vv+ viruses at the high end of the continuum and the nv (non-virulent) MDV vaccine strains, this classification based on their abilities to resist immunities induced by different types of vaccines (Morrow *et al.,* 2004; Nair *et al.,* 2004; Nair, 2005; Murata *et al.*,2013).

2.3 Epidemiology of Marek's Disease

Marek's disease exists in poultry-producing countries throughout the world (Witter and Schat, 2003). Although various MD outbreaks are occasionally reported in the literature, in general it is difficult to ascertain the global status of MD (Dunn and Gimeno, 2013). The following are suggested as possible reasons:

1. Marek's disease is not a notifiable disease.
2. Low level losses after MD vaccination are generally accepted and treated as normal, since it is known that vaccination failures occur at low frequency.

3. Occurrence of MD is often linked to financial claims between rearing companies and hatcheries or hatcheries and vaccine manufacturers. Often such cases are not made public.

4. Since prevention of the disease requires optimal hygiene and management, besides several other measures, many MD cases are not reported in order to avoid damaging the reputation of the companies/farms concerned (Morrow *et al.*,2004).

In a survey conducted using questionnaires in 2003, 45% of the 55 countries reported severe outbreaks in the previous 10years, while a similar survey conducted in 116 countries in 2011, shows that many parts of the world particularly French speaking Africa, Eastern Europe, east Asia and Australia reported frequent diagnosis of MD in their countries(Gimeno, 2004; Dunn and Gimeno, 2013). In addition the survey shows that at least one respondent from 89% of countries reported MD is either occasionally or frequently diagnosed, with respondents from 75 countries indicating frequent and those from 29 countries reporting occasional diagnosis. While respondents from nearly half of the countries (48%) reported that MD incidence increased during the last 10 years (Dunn and Gimeno.,2013).

Many factors have been postulated as reasons for increasing incidence of MD, after the boom of the poultry industry in the 1960s, when the poultry production was not on an intensive scale, both the virus and the hosts were able to achieve a state of balanced co-existence. However, the transformation of the poultry industry into the intensive production practices from the early 1960s saw a shift in this balance greatly in favour of the virus. The continuous availability of large populations of genetically susceptible naive hosts, usually in an overcrowded environment, enabled the virus to spread rapidly,

encouraging their rapid evolution towards greater virulence. This was evident when huge MD outbreaks swept through poultry flocks in the 1960s, wiping out large populations all around the world (Marrow *et al.,* 2004; Nair, 2005). Other factors include coexistence of other immunosuppressive diseases, short rest period and downtime, poor cleanout, increased multi-aged farms, poor biosecurity, inappropriate nutrition, low Plaque forming unit levels of vaccines, poor vaccine administration or handling, vaccine failure, missed vaccination, inappropriate vaccine, e.t.c (Dunn and Gimeno, 2013).

2.3.1 Host of Marek's disease

Chickens are by far the most important natural host for MD, but quails, turkeys, and pheasants are also susceptible to virus infection and disease. Virtually all chickens including gamefowl and jungle fowl are susceptible to MDV infection and tumor development (Witter and Schat, 2003). Marek's disease virus serotype 1 has been demonstrated in migratory and sedentary waterfowl (Murata *et al.,* 2011). The outcome of the disease is influenced by sex, usually females birds are more susceptible than males (Payne *et al.,* 2000).

2.3.2 Transmission of Marek's disease virus

Marek's disease virus is transmitted only horizontally, transmission readily done by direct or indirect contact between chickens, apparently by the airborne route (Davidson *et al.,* 2002; Witter *et al,* 2003; Islam *et al.,* 2007). Marek's disease virus serotype 1 and 2 are contagious, but HVT does not spread readily between chickens infected early in life (Islam *et al.,* 2007). Natural infection is believed to begin through inhalation of virus, epithelial cells in the keratinizing layer of the feather follicle replicate fully infectious virus, and these cells serve as a source of contamination to the environment

and to other chickens (Witter *et al,* 2003; Jarosinski, 2012). Virus associated with feathers and dander is infectious, and contaminated poultry house dust remains infectious for at least several months at 20—25°C and for years at 4°C . Dander is assumed to wrap the cell free virions and to protect them physically from degradation, which explains the higher resistance of MDV virions in the environment compared with other herpesviruses (Denesvre, *et al.,* 2013).Under commercial field conditions, young chickens are most commonly exposed to MDV by contact with residual dust and dander in the growing house or by the introduction of these materials by aerosols (from adjacent chicken houses), fomites, or personnel. After the virus is introduced into a chicken flock, regardless of vaccination status, infection spreads quickly from bird to bird. Virus excretion begins about 2 weeks after inoculation or exposure, with maximal shedding occurring between the third and fifth weeks. Once infected, chickens appear to shed virus indefinitely (Witterand Schat.,2003). The MDV replicates productively in feather follicle epithelium cells and enveloped virions are shed from this site hence serving as the virus assembly site, feathers contain massive amounts of cell-free virus which are the source of infection and perpetual dissemination of the virus throughout the life time of the infected bird (Borenshtain and Davidson, 2002; Baigent *et al.,* 2013).

2.3.3 Incubation period of Marek's disease

The incubation period for MD with observation of mononuclear infiltrations found in nerves and other organs is about 2 weeks. Clinical signs and gross lesions, however, generally do not appear until after 3-4 weeks (Witter and Schat, 2003).

The clinical expression of both acute and classical forms of transient paralysis usually occurs from 8-18 days post infection. Field cases of transient paralysis are seen mainly

between 6-12 weeks of age, probably reflecting recent MDV exposure, 8-10 days prior to the onset of clinical signs (Witter and Schat.,2003).

2.4Evolution of Marek's Disease Virus

2.4.1 Pathogenesis of Marek's disease

Pathogenesis of Marek's disease can be divided into four phases: an early cytolytic phase (2-7 days post-infection, or dpi), a latent phase (7-10 dpi onwards), a late cytolytic and immunosuppressive phase (18 dpi onwards) and a proliferative phase (28 dpi onwards) (Witter and Schat, 2003; Baigent*et al.*, 2004).

2.4.1.1Early cytolytic phase

Natural infection is believed to begin through inhalation of virus, the phagocytic cells in the respiratory tract i.e the macrophages become infected either directly or after an initial round of replication in epithelial cells. Within 24 hours of uptake, virus is detectable in the spleen, thymus and the bursa of Fabricius (Calnek, 1998;Osterrieder *et al.,* 2006; Jarolski *et al.,* 2012). The bursa has been shown to provide an environment for this critical phase of MDV replication cycle(Abdul-Careem *et al.*, 2008). Here, the virus meets its primary targets for the first phase of cytolytic replication: B cells and later activated CD4+ and, rarely, CD4–CD8– T cells or CD8+ T cells. The peak of virus replication in these cells is observed between three and seven days post infection (Osterrieder *et al.,* 2006). It is generally accepted that resting T cells are fairly refractory to infection but that cytolytic infection of B cells induces activation of T cells, rendering these cells susceptible to infection (Baigent *et al.*, 2004; Nair, 2005). Infected CD4+ T cells not only serve as a target for transformation and as the reservoir for latent MDV genomes, they are also the means of virus spread within an infected animal, and allow

transport to the skin. Infected T cells appear to be the route by which MDV enters the feather-follicle epithelium, where free infectious virus assembles and is shed to infect chickens that come into contact with the infected bird (Osterrieder *et al.,* 2006).During this period a cytolytic infection, often very severe, ensues in the lymphoid organs leading to lymphoreticulitis and enlargement of the spleen, and necrosis and atrophy of the bursa and thymus with accompanying inflammatory changes. A cell-associated viraemia also develops (Calnek, 1998; Nair, 2005).

A hallmark of lytic MDV infection is substantial and sustained downregulation of major histocompatibility complex (MHC) class I molecules on the surface of infected cells. The downregulation is encoded by an MDV early gene(s) and is maintained throughout the lytic cycle, resulting in virus-infected cells that are 'invisible' to the cytotoxic immune response carried out by CD8+ T cells. It is currently surmised that escape from MHC class I restricted cytotoxicity facilitates the establishment of, and reactivation from, latency and is important forvirus dissemination. This escape allows MDV to reach epithelia in the liver, lung, kidneys, oesophagus, proventriculus (glandular stomach), adrenal gland and skin, organs where productive infection can be established (Osterrieder *et al.,* 2006).

2.4.1.2 Latent Phase

Marek's disease virus enters the latent phase of infection from approximately 7 days p.i. Latency is defined as the presence and maintenance of viral genomes without production of infectious progeny virus (Osterrieder *et al.,* 2006).There are three stages of MDV latency: establishment, maintenance and reactivation, but the virus-host relationship is dynamic and transition between these states can occur (Baigent *et al.,*

2004). Many host factors may determine the switch from cytolysis to latency, firstly latency in T and B cells is broken at 12-30hours after explantation so that they are free from host influences and secondly latency are observed when initial immune responses are evident 6-7 dpi. Studies have also shown or supported the view that host immune responses are crucial for the establishment and maintenance of latency (Baigent *et al.,* 2004).

During this period, the virus is also carried by the infected lymphocytes to the skin where a fully productive infection occurs in the feather follicle epithelium; infectious cell-free virus is shed from about 10 days after infection independent of the genetic resistance of the host (Baaten *et al.,* 2004; Nair, 2005). In susceptible birds, the latently infected T cells become targets for neoplastic transformation resulting in the development of lymphomatous tumours in various visceral organs (Nair, 2005). Interestingly, clinical signs of the infection diminish, productive viral antigen cannot be detected in the bursa, thymus and spleen, and within 2 weeks the lymphocyte populations in the bursal follicles and thymic cortex return to normal (Baaten *et al.,* 2004).

2.4.1.3 Late Cytolytic and immunosuppressive phase

*I*nflammatory changes as mention in the latency stage resolves 7-14 dpi, however in susceptible birds there is a second wave of cytolytic infection this is coincident with permanent immunosuppression and Iymphoma development (Baiegnt *et al.,*2004). This stage occurs in lymphoid organs with localize foci of infection in the kidney, Pancreas, adrenal gland and proventriculus (Witter *et al.,*2003) . There severe atrophy of the Bursa

and thymus, necrosis of lymphocytes with accompanying inflammation as well as infiltration of mononuclear cells and heterophils (Baigent *et al.,* 2004).

2.4.1.4 Development of lymphomas

The most important and obvious outcome of infection with MDV is transformation of infected cells followed by multifocal lymphoma formation, death from lymphomas may occur at any time from about 3 weeks onward (Witter and Schat, 2003; Osterrieder *et al.,* 2006). Lymphomas can involve nearly any tissue of the body, but are particularly prominent in visceral organs, peripheral nerves, muscle and skin (Calnek, 1998). Neoplastic transformation of individual lymphocytes leading to the appearance of microscopic and gross lymphomas is clearly a rare event; otherwise, virtually every infected chicken would soon die of massive tumours given the number of infected cells in a chicken exposed to the virus. On the other hand, given the right conditions, e.g., genetic susceptibility and a highly virulent MDV strain, the probability of tumour development is extremely high; often times the incidence can reach 100%. In birds destined to succumb to tumours, gross lesions may appear as early as 2 to 3 weeks post-infection or in some cases as late as several months after exposure (Calnek, 1998).

The composition of lymphomas is complex, consisting of a mixture of neoplastic, inflammatory, and immunologically committed and non-committed cells. Both T and B cells are present, although the former predominate, with about 75% observed to be T cells while 15% are B cells (Witter and Schat, 2003; Baigent *et al.,*2004). From 3--4 weeks post-infection, non-productively infected lymphocytes progressively migrate into the visceral organs and peripheral nerves, where, under the influence of as yet undetermined factors, they proliferate to form lymphomas. Lymphocytes continually

circulate from the blood to the lymphatics via lymphoid and non-lymphoid tissue, and migration through endothelial cell monolayers requires the production of matrix-degrading enzymes- an ability that increases in activated T cells, such as those latently infected with MDV (Baigent *et al.,* 2004).

2.5 Immunity Against Marek's Disease Virus

Marek's disease virus (MDV) causes debilitation of the immune system and immunosuppression thereby making the host vulnerable to infection by all other pathogens (Davison *et al.,* 2004). All chickens are susceptible to MDV infection, and vaccination is essential to protect the susceptible host from developing clinical disease. Nevertheless, MDV infects and replicates in vaccinated chickens, with the challenge virus being shed from the feather-follicle epithelium (Davison *et al.,* 2004). Several host factors are attributed to influence the course of MD, predominantly the responses of the innate and acquired immune systems, and these are modulated by: age at infection and maturity of the immune system; vaccination status; co-infection with other (immunosuppressive) pathogens; the sex of the host; and various physiological factors (Davison *et al.,* 2004). The immune responses during the early cytolytic phase of infection are crucial for the outcome of MDV infection. Impairment of immune responses during this phase delays establishment of latency resulting in prolonging the lytic infection and the subsequent continued destruction of immune cells by virus-induced apoptosis. Impairments include infection at 1 day of age when the immune responses are not yet fully developed or treatment with cyclosporin or neonatal thymectomy combined with cyclophosphamide treatment. The importance of immune responses during latency is also very relevant for protection against the second cytolytic phase and is dependent on cell-mediated immunity(Witter and Schat, 2003).Effective

immunity to MD requires involvement and coordinated activation of innate defence mechanisms and adaptive immune responses (Haq *et al.*,2013).

2.5.1 Innate host responses

Innate immune responses are rapidly induced by viral infection, controlling early events until acquired immune responses become fully effective several days later (Davison *et al.,* 2004). Birds are infected with MDV by inhalation of the infected poultry dust and dander shed in form of flakes of skin from infected birds although the actual site(s) and cellular mechanism involved with uptake have not yet been clearly identified, most research that have involve challenge with the virus have been inoculation of the virus parenterally (Davison *et al.,* 2004.; Haq *et al.,* 2013). The innate immunity involves the interferon, macrophages and Natural killer cells.

2.5.3 Macrophages

In as much as Macrophages have a number of essential roles in MDV infection, they also play a pivotal role in innate immunity and the development of adaptive immune responses by acting as antigen-presenting cells (Davison *et al.,* 2004). Macrophages inhibit MDV replication by inhibition of MDV DNA synthesis and play an important role in the reduction of viremia in the early cytolytic phase of MDV infection, hence reducing the likelihood of clinical MD development, but their role in the tumour phase is not understood. But it has been suggested that Macrophages inhibit the proliferation of tumours cells, and uncontrollable proliferation T-cells which is ultimately beneficial to the host (Powell, 1985; Davison *et al.,* 2004; Haq *et al.,* 2013). Another method of antiviral activities by Macrophages is through the production of mediators such as Nitric Oxide (NO). Research has shown that higher concentration of NO result in greater inhibition of MDV replication (Haq *et al.,* 2013).

2.5.4 Natural killer cells

Natural Killer (NK) cells are an important population of innate immune cells that are non-adherent and non-phagocytic lymphocytes and are fundamental in defences against cytopathic viruses, particularly herpesviruses, and tumour cells (Davison *et al.,* 2004). NK cells are the first line of defense, lyse virus-infected and tumor cells without prior exposure to the pathogen which is done via the induction of apoptosis. This may be achieved using one of two pathways: ligation of cell death receptors and release of granular contents, e.g., granzyme and perforin which are a serine protease and a pore-forming protein, respectively (Witter and Schat, 2003; Haq *et al.,* 2013). Several research studies have shown an increase in the activity of NK cells in MD-resistant and vaccinated chickens when compared to unvaccinated and MD-susceptible chickens. In addition in vitro study also demonstrated NK-like activity against MD tumor cells, suggesting that NK cells may play a role during the transformation phase and can target MDV-transformed cells, which may suggest that NK cells have an important role in protective immunity against MDV and are likely to be most active in the early cytolytic phase of infection (Chen *et al.,* 2013; Haq *et al.,* 2013).

2.5.5 Interferon

Interferon-gamma (IFN-c) is a type II interferon and an important regulator of immune responses including antiviral defenses. Chicken IFN-c (chIFN-c) was first cloned in 1995, and since then has been shown to activate macrophages, neutralize viral replication, enhance major histocompatibility complex (MHC) class II antigen expression and other genes in chickens. The expression of IFN-c is associated with protection against MD making this cytokine a candidate for inclusion in Marek's disease vaccines to enhance their protective efficacy (Haq *et al.,*2011).

2.5.6 Adaptive immune responses

The adaptive immune response, which comprises cell-mediated immune responses and Humoral immune response(neutralizing antibodies), also plays a significant role in antiviral immunity against herpesviruses. These immune responses are antigen-specific, and involve secretion of antibodies against various MDV proteins, in addition to cell mediated immune responses initiated by the interaction of CD4+ and CD8+ T cell receptors (TCR) with the MHC molecules loaded with MDV antigens (Schat and Xing, 2000; Haq *et al.,* 2013).

2.5.7 Humoral immunity

Due to the cell-associated nature of MDV antibodies are of limited importance in MD immunity or not clearly known but passively and actively acquired antibodies have been implicated in protective immunity against MDV (Witter and Schat, 2003;Davison and Kaiser, 2004;Haq *et al.,* 2013).

Chickens infected with MDV develop precipitating and viral neutralizing antibodies within 1—2 weeks; a transient immunoglobulin M (IgM) response is replaced by IgG .These antibodies are made against a large number of proteins. Most of the antibodies are not relevant for protective immune response, because they detect either nonstructural proteins or proteins that are not expressed in the virus envelope or on the surface of virus-infected cells(Witter and Schat, 2003). Antibodies are believed to play a role in immunity against the virus by neutralizing cell-free viruses, blocking entry of virus into cells, and antibody-dependent cell-mediated cytotoxicity (ADCC) (Haq *et al.,* 2013).

2.5.8 Role of antibodies

2.5.8.1 Maternal antibodies

Acquires maternal antibodies from the vaccinated or MDV infected hen delay the development of clinical signs of MD, providing some protection against MD morbidity, tumour formation and mortality by delaying in virus replication (Davison *et al.*,2004, Schat and Xing, 2000). Although the presence of maternal antibodies has deleterious effect on vaccine efficacy, particularly cell-free HVT vaccines, administered either *in ovo* or to the neonate because of their neutralizing action on the free vaccine virus (Schat and Xing, 2000; Haq *et al.*, 2013). Maternally derived antibodies are loss after three weeks of age (Witter, 1998; Payne, 2004).

2.5.8.2 Roles of cell mediated immune responses

Cell-mediated cytotoxic responses are critical for control of Marek's disease virus (MDV) infection and tumour development (Chen *et al.*, 2013). Anti-viral immunity against herpesviruses is principally mediated by Cytotoxic T lymphocytes (CTL) and CD4 + helper cells that secrete cytokines, which is because of the lymphotropic and cell associated nature of the virus (Witter and Schat, 2003; Davison and Kaiser, 2004). It was suggested that CTL responses is important for the elimination of MDV- infected cells and research findings has shown that CTL may play a role in genetic resistance to MD (Schat and Xing, 2000; Haq *et al.*, 2013).

Cytokines have been shown to be important mediators involved in cell-mediated immune responses to MDV infection and are secreted as a result of antigen presentation to T cells they may also play a part in vaccinal immunity (Haq *et al.*, 2013).

2.5.9 Immune regulation by Marek's disease virus

Herpesviruses have evolved several effective strategies to counter the host immune response. Chief among these are inhibition of the host MHC class I antigen processing and presentation pathway, thereby reducing the presentation of virus-derived epitopes on the surface of the infected cell, some other mechanisms can lead to general or local down-regulation of the host immune system or immunosuppression (Schat, 2004; Griffin *et al.*,2010; Haq *et al.*,2013). The hallmark of herpesvirus infections is the ability to establish latency in the host after the primary lytic infection. Latency is the optimal host-pathogen relationship: virus is present in the host without producing proteins that are recognized by immune responses (Schat and Xing, 2000). Several herpesviruses have been reported to code for proteins which play a role in the immune responses such as v-IL-6, IL-10, IL-17, interferon response elements hence the immune may not be able to recognize the antigen as foreign to the body (Schat and Xing, 2000). Research has also shown that herpesviruses are able to produce proteins resembling MHC-I to as serve decoy to foil Natural Killers cells, which attack target cells lacking or with decreased levels of MHC-I antigen expression(Schat and Xing, 2000). Another mechanism of immune evasion by MDV is through apoptosis, MDV delays apoptosis in T- lymphocytes, Apoptosis of virus-infected cells can be beneficial to the host, if cells are eliminated prior to virus assembly or detrimental to the host, if programmed cell death facilitates the release of mature virus particles. It is therefore not surprising that MDV code for proteins inhibiting apoptosis during the early phase of replication but facilitate the process during the later stages of replication which is beneficial to the virus (Schat and Xing, 2000).

2.5.10 Immunosuppression

Immunosuppression can defined as 'a state of temporary or permanent dysfunction of the immune response resulting from insults to the immune system and leading to increased susceptibility to disease', as originally proposed by Dohms and Saif (Schat, 2004). Suppression of the immune response by MDV infection is a critical feature of the disease, contributing to the virulence of MDV isolates and altering susceptibility of the host to other pathogens (Witter and Schat, 2003). Marek's disease associated immunosuppression is often divided into an early immunosuppression phase, late immunosuppression phase (Schat, 2004; Schat *et al.*, 2008; Haq *et al.*,2013).

2.5.10.1 Early immunosuppression

The key characteristics of this phase are lymphocytes destruction, resulting in thymus and/or bursal atrophy, and macrophage activation which inhibit mitogen stimulation of T cells. Down-regulation of various surface molecules, such MHC class I, on lymphocytes also contribute to immune evasion strategies used by MDV (Heidari *et al.*, 2010; Haq *et al.*, 2013). Destruction of B and T lymphocytes has direct negative impact on the chicken adaptive immune responses that consequently leads to severe immunosuppression; apoptosis is the likely mechanism of MDV-induced lymphocyte destruction (Heidari *et al.*, 2010).

2.5.10.2 Late Immunosuppression

Late immunosuppression can be caused by lytic infection of lymphoid cells upon reactivation of MDV from latency, causing similar effects on the immune system as during the early lytic infection (Schat *et al*., 2008).

MDV causes immunosuppression through loss of lymphocytes as a result of virus replication, virus-induced changes in the regulation of immune responses and tumour cell-induced immunosuppression (Schat, 2004).

2.5.11 Vaccination against Marek's disease

Vaccination against MD was introduced in 1970 using the herpes virus of turkeys (HVT) which then provided excellent protection against the disease (Baigent *et al.,*2006). Marek's disease vaccines were the first successful anti-cancer vaccines to be developed, and a number of other features make them unique in the field of vaccinology (Bublot *et al.,* 2004). These unique features about MD vaccines are as follows:

1. Most are sold as frozen cell-associated stocks requiring liquid nitrogen for transport and storage.

2. MD vaccines should provide protection against very early challenge (within the first days of life), and therefore are administered at hatching or *in ovo.*

3. A single administration should induce lifelong protection.

Marek's disease vaccines have brought enormous benefits to poultry production and without which the modern poultry industry would not have survived the scourge of MD and be able to become so productive. Almost all layers and breeders are vaccinated against MD and in some countries where MD is a problem; broilers are also vaccinated (Bublot *et al.,* 2004).

The use of MD vaccines has some drawbacks. The most important one is their inability to induce a sterile immunity in the vaccinated host. This allows the virulent virus strains

to replicate and be shed into the environment in spite of the vaccination status of the host (Nair, 2005). In other words MD vaccines do not prevent super-infection by challenge viruses, multiplication of the virulent challenge virus and its shedding from feather tissues into the environment still occurs (Nair, 2005). This has two major consequences. First and foremost, virulent virus shed by vaccinated birds is still oncogenic to non-vaccinated birds. Secondly, the continued evolution of field viruses towards greater virulence is attributed to the selection pressures imposed on these virulent viruses in vaccinated birds. An ideal vaccine would be one that prevented replication of the virulent virus (sterilizing immunity), or at least its shedding. (Witter, 1997; Witter *et al.*, 2005, Baigent *et al.*, 2006; Hunt *et al*.,2013). Consequently, most poultry houses are infected with MDV as a result of the failure of the MD vaccine to confer sterilizing immunity on the birds, presently no sterilizing vaccines have been developed for MD and the prospect of developing one anytime soon may be slim despite all the volume of research work going on in this area of the disease (Bublot *et al.*, 2004; Hunt *et al*., 2013).

Revaccination is a common practice in the control of MD and it is advantageous if done at day 18 in ovo in embryonated eggs followed by day 1 using HVT and CV1 988 or HVT+SB1 at 18ED and CVI 988 at day 1(Tan *et al.*,2007; Gimeno *et al.*,2012). This practice was reported by Gimeno, (2004) in a surveyed carried out in 2003 which involved 55 countries. The protocol included vaccination in ovo at 18 days followed by revaccination at 4-7 hours after hatching or at 7, 18 or 21 days of age. Herpes virus of turkey is commonly used in layers and breeders, while in endemic environments broilers are also vaccinated. For best protection vaccines should be administered at 18 ED a better protective vaccine at 1 day old (Gimeno *et al*., 2012)

2.5.11. 1Type of Marek's disease vaccines

Several different types of MD vaccines are in common use, both individually and in various combinations. The most widely used products are attenuated serotype 1 MDV, naturally nonpathogenic HVT and serotype 2 viruses (Witter and Schat, 2003).

2.5.11.2 Herpes virus of turkey vaccine serotype 3 (FC 126)

The most widely used strain is the FC-126 strain developed in the year1970, it is use both as cell free lyophilized vaccine or as cell associated vaccine. Although research has shown that cell-associated HVT was several-fold more effective than cell-free HVT vaccine in chicks with maternally derived antibodies at the time of vaccination (Bublot *et al.,* 2004; Biggs *et al.,* 2012). This MD vaccine can only prevent infection against mMDV and vMDV (Payne, 2004). In a survey carried out in 2003, it was noted that over 27 countries around the world that HVT is used to vaccinate broilers, it is the most preferred vaccines and continue to be used around the world despite more effective vaccines that are available, this may be due to cheaper cost of storage in the cell free type (Witter *et al.,* 1998; Gimeno, 2004). HVT is used to in combination with SB-1 strain and most commonly with CV1988 serotype 1 strain to vaccinate birds in order to achieve better results (Gimeno, 2004).

2.5.11.3 Marek's disease Serotpe 2 vaccine (SB-1)

In response to the increasing number of MD outbreaks in vaccinated flocks in the 1980s and the increasing ineffectiveness of the HVT, new vaccines, based on the non-oncogenic serotype-2 MDV were introduced, usually in bivalent combination with HVT (Witter, 1998; Baaten *et al.,* 2004). They are apathogenic strains isolated from clinically normal birds and prevent infection with vvMDV particularly as bivalent

vaccines, and the other pathotypes that are preventable by the HVT vaccines (Payne, 2004; Gimeno, 2008). The first serotype 2MDV strain that was licensed to be used as a vaccine was strain SB-1 which was introduced in the Mid 1980s (Singh *et al.,* 2010). The level of protection conferred by serotype 2 vaccines administered alone is low but they exhibit protective synergism when used in combination with serotype 3 strains in bivalent vaccines than with either serotypes administered alone(Gimeno, 2008, Singh *et al.,* 2010). Furthermore, compared with other vaccines, SB-1 has been reported to give very good protection in maternal antibody-positive chicks (Singh *et al.,* 2010). Clone SB-1 have been shown to lower replication and shedding of MDV compared to SB-1,both vaccines reduced replication and shedding of RB-1B, and were equally effective in protecting chickens against MD(Singh *et al.,* 2010).

2.5.11.4 Marek's disease Serotpe 1 vaccine (Rispens)

These are attenuated MD vaccines, the first MD vaccines used was the HPRS-16 which required 33 passages in chicken kidney cell cultures although this has been replaced by the Rispens CV1988 strain (Tan *et al.,* 2007; Gimeno, 2008). The CVI988 strain had low oncogenic potential upon initial isolation but was attenuated by 26–35 serial passages in duck embryo fibroblast culture. The CVI988 vaccine strain seems to have an extraordinary ability to protect chickens against challenge with highly virulent MDV and has become the 'gold standard' providing superior protection against highly virulent challenge strains of MDV (Bublot, 2004; Tan *et al.,* 2007;Gimeno, 2008; Baigent*et al.,* 2011).

2. 5.11.5.Marek's disease polyvalent vaccines

There are many combinations of MD vaccines: SB-1 strains (serotype 2) and the FC126 strain (serotype 3, HVT), HVT and Rispens CV1988. This was prompted by the emergence of very virulent MDV in the 1980s (Ross,1998;Gimeno, 2008). Combinations of the SB-1 strains (serotype 2) and the FC126 strain (serotype 3, HVT) provide better protection than either vaccine alone which is known protective synergism, is the basis for the bivalent vaccines developed in the 1980s. Synergism is strongly serotype specific and is especially evident between combinations of serotypes 2 and 3. No protective synergism has been reported between combinations of serotype 1 with serotypes 2 or 3. However, augmentation of protection for MD vaccines has been observed with two additional systems like the combinations of HVT and glutaraldehyde-inactivated serotype 1 MDV (Gimeno, 2008).

2.5.11.6 Vaccine induce immunity

One of the mechanisms of induction of immunity by MD vaccine is through reduction of growth rate of the pathogenic MDV,it is a well-documented fact that vaccinated birds shed fewer viruses than unvaccinated birds (Nair, 2005; Islam *et al.*,2007). Several published works has shown that shedding rate of MDV 1 in vaccinated flocks is half that of unvaccinated flocks although the vaccine virus is also shed into the environment (Islam *et al.*, 2007). MDV serotype 2 has the highest shedding rate compare to the other serotypes, which may have limiting effect on the shedding rate of MDV 1. Virus shedding rates have been associated with the virulence of MDV1 strains (Islam *et al.*, 2007). Vaccination with HVT and CVI988 vaccine viruses elicit immune response in the feather follicle epithelium of the host characterized Interferon γ and $CD8^+$ cell infiltration which may be used to reduce viral shedding from site of infection (Abdul-

Careem *et al.*, 2008). CVI988 elicit maximal protection between 1-2 weeks after vaccination and exposure to the virulent strains, furthermore greater protection is attributed to more replication in the spleen rather high levels of latent virus, although vaccine virus with high levels of replication tends towards virulence (Baigent *et al.*,2005). MD vaccines targets MDV replication in the cytoiytic phase and prevent lymphoma development, they stimulate innate responses, antibodies production and cell mediated responses(Davison *et al.*,2004;Singh *et al.*,2010).

Even though the MD revaccination strategy has become common practice in many countries with serious problems with MD, the mechanisms by which superior immunity is induced by revaccination is not known up till recently(Gimeno, 2004;Wu *et al.*,2008). Revaccination of the virus induced in the chickens a higher and a longer temporary expansion of the $CD8^+$, $CD4^+$, and $CD3^+$ T-lymphocyte subpopulations, stronger peripheral blood lymphocyte proliferative activity; and higher levels of neutralizing antibody than single vaccination. These findings disagree with the postulate that MDV antigens persist, stimulate the immune system, and maintain a high level immunity after vaccination (Wu *et al.*, 2008).

2.6 Clinical Signs of Marek's Disease

Signs associated with MD vary according to the specific syndrome. Chickens withMD lymphoma or fowl paralysis syndromes may exhibitsigns, but few are specific to MD (Witter and Schat, 2003). In general, signsrelated to peripheral nerve dysfunction are those associatedwith asymmetric progressive paresis, spastic or flaccid, eventually culminating in complete paralysis (Purchase, 1985, Witter and Schat, 2003). Almost any nerve in the body may be involved unilaterally or bilaterally. An uneven gait, lameness, favoring of one leg over the other, and paralysis of one leg or both wings also occurs.

Involvement of the motor nerves to the neck muscles may result in the head being held low and torticollis (Purchase, 1985). Involvement of the vagus nerve can result in paralysis anddilation of the crop and/or gasping. Because locomotorydisturbances are easily recognized, incoordination orstilted gait may be the first observed sign. A particularlycharacteristic clinical presentation is a bird with one legstretched forward and the other back as a result of unilateralparesis or paralysis of the leg (Purchase, 1985; Witter and Schat, 2003).

In affected flocks, birds appear unthrifty, have delayed feathering, have ruffled feathers, and are stunted, in addition other non-specific signs may be seen which include weight loss, paleness, anorexia, and diarrhea may be observed, especially in birds in which the course is prolonged. Under commercial conditions, death often results from starvation and dehydration because of the birds' inability to reach food and water or, in many cases, from trampling by flock mates (Purchase, 1985; Witter and Schat, 2003). Birds with ocular involvement may show evidence of blindness, which can be unilateral or bilateral, although recognition of clinical blindness requires careful observation. The blindness is associated with lymphoid infiltration of the Iris leading to iris changing colour from reddish brown to gray and usually the birds cannot stand intensity of light (Purchase, 1985;Charlton *et al.,* 2000; Witter and Schat, 2003). The effects of tumors are sometimes seen at the base of the comb and on featherless parts of the hock and shank resulting in roughening of the scale-covered skin as well as lumps between the toes. On palpation, feather enlarged follicles or occasionally, tumors may be felt on the feather parts of the body (Purchase, 1985). Immunosuppressed chickens may succumb to secondary infections which may mask the MD, but some chickens' die 20—40 days post infection with few signs (Purchase, 1985; Witter and Schat, 2003). Clinical signs

usually do not appear prior 3weeks of age and peak between 2-7 months, although mortality as result of MD has reported in one week old naturally reared non- vaccinated chickens. (Charlton *et al.,* 2000;Carvallo *et al.,* 2011).

2.7 Gross Pathology of Marek's Disease

The gross changes commonly present in the classical and acute MD are enlargement of the peripheral nerves, lymphoma formation in various organs and tissues, and atrophy of the bursa of fabricius and thymus(Payne, 1985; Witter and Schat, 2003).

2.7.1 Nerves

Enlarged peripheral nerves are present in almost all the chickens with classical signs of MD and in many chickens with acute form of the disease (Payne, 1985). The nerves are usually two or three times the normal size with loss of cross striations and gray or yellow discoloration, and sometimes an edematous appearance (Payne, 1985; Charlton *et al.,* 2000; Witter and Schat, 2003). The mostly commonly affected nerves are the brachial and siatic plexus trunks, celiac plexus, abdominal vagus and intercostal nerves(Payne, 1985).

2.7.2 Visceral organs

Lymphomas may occur in one or more of a variety of visceral organs and tissues. Lymphomatous lesions can be found in the gonad (especially the ovary), lung, heart, mesentery, kidney, liver, spleen, bursa, thymus, adrenal gland, pancreas, proventriculus and intestine (Witter and Schat, 2003). Multiple involvements of visceral organs are usually in the acute form of the disease while it is less common in the classical form of the disease. In the classical form of the disease the lymphomas are small, soft grey

tumours in the ovary, kidney, heart, liver and other tissues (Payne, 1985, Payne*et al.*,2000). MD lymphomas in most viscera appear as diffuse enlargements, sometimes to several times the normal size, and a diffuse white or grayish discoloration is often present. Alternatively, lymphomas may occur as focal, nodular growths of varying size. Nodules are white or gray in color and are firm, and the cut surface is smooth (Witter and Schat, 2003). The liver may be moderately or greatly enlarged with miliary or nodular lesions(Payne *et al.,* 2000). Diffuse infiltration of the liver causes loss of normal lobule architecture and often gives the surface a coarse granular appearance (Witter and Schat, 2003).

2.7.3 Ovaries

With large tumors, the normal foliated appearance of the ovary is obliterated. Mature ovaries may retain function, even though some follicles are tumorous. Marked involvement is indicated by a cauliflower-like appearance (Witter and Schat, 2003).

2.7.4 Proventriculus

Here the proventriculus becomes thickened and firm as a result of focal leukotic areas within and between the glands, which may be seen through the serosal surface or, if involvement is diffuse, detected by palpation(Witter and Schat, 2003).

2.7.5Skin

The lesions seen in the skin are nodular lesions involving few scattered follicles, or they may be numerous and coalesce. The distinct whitish nodules, especially evident in dressed carcasses, may become scablike with brownish crust formation in extreme cases (Witter and Schat, 2003).

2.7.6 Eyes

Gross ocular changes include loss of pigmentation in the iris ("gray eye") and irregularity of the pupil, both the result of mononuclear infiltration of the iris (Witter and Schat, 2003). Conjunctivitis, occasionally with multifocal hemorrhages and corneal edema has also been reported (Ficken *et al.*, 1991).

2.8 Histopathology of Marek's Disease

Regardless of their anatomical location, MD lesions have common fundamental histopathological features. Early and/or mild pathology is distinguished by perivascular mononuclear cell 'cuffing' from 5-7 days post-infection (dpi)- during the cytolytic phase of infection- suggesting both increased vascular permeability and leukocyte adhesion with extravasation (Burgress, 2004). Microscopically, the lymphomas are characterized by a mixture of Pleomorphic lymphocytes, a few of which are probably true tumour cells that carry T-cell surface antigens and a tumor-associated antigen (MATSA) as well as B- and T-cells (Charlton *et al.*, 2000).

2.8.1 Nerves

Cellular infiltration of peripheral nerves, leading to their enlargement and paralysis is a characteristic feature of classical MD (Bugress, 2004). In peripheral nerves, 2 main types of lymphoproliferative lesions are recognized. One type is considered neoplastic, consisting of a mixed population of small, medium and large (blastic) lymphocytes, activated reticulum cells and macrophages, in some cases, demyelination and Schwann cell proliferation are associated with this lesion (Payne, 1985; Witter and Schat, 2003; Payne, 2004). The second type is essentially inflammatory and is characterized by diffuse, light-to-moderate infiltration by small lymphocytes and plasma cells, usually

with edema, and sometimes, with demyelination and Schwann cell proliferation (Payne, 1985; Witter and Schat, 2003).

2.8.2 Visceral organs

Lymphomatous lesions in visceral organs are more uniformly proliferative in nature than those in nerves. Cellular composition is similar to that of the proliferative lesions described for nerves, consisting of diffusely proliferating small-to medium lymphocytes, lymphoblasts, and activated and primitive reticulum cells (Payne, 1985; Witter and Schat, 2003; Payne, 2004). Macrophages are also present in the tumor mass, especially in slow-growing tumors, perhaps reflecting the host immune response. The cellular composition of tumors is similar from one organ to another, even though the gross pattern of involvement may vary (Witter and Schat, 2003).

2.8.3 Skin

Skin pathology has two components; lytic infection of the FFE and perivascular accumulation of lymphocytes in the dermis. Perivascular lymphocyte accumulation occurs from 5-7 dpi. If these lymphocyte accumulations increase in size and become visible to the naked eye, they are termed 'skin leukosis' (Payne, 1985; Burgess, 2004).

2.8.4 Ocular changes

Common findings in the eyes are lymphocytic, plasma cell and sometimes heterophil infiltration of the iris, ciliary body and conjunctiva, and less commonly of the choroid membrane and retina (Payne, 2004). In MD cases with blindness, a common feature is extensive infiltration by inflammatory cells in the cornea, uveal tract, retina and pecten, intranuclear inclusion bodies in mononuclear cells and retinal cells, and degeneration of photoreceptors and other retinal cells (Ficken *et al.*, 1991; Payne, 2004).

2.9 Diagnosis of Marek's Disease

Despite the success of vaccination, widespread outbreak of MD still occurs in commercial flocks worldwide hence the need for prompt, accurate and rapid diagnosis of the disease is needful (Zelnik, 2004). Primarily diagnosis is based on age clinical signs, history, gross and microscopic lesions (Singh *et al.*, 2012). While in the laboratory diagnosis is by numerous virological techniques such as viral isolation, identification of MDV-specific antigens or antibodies as well as viral Nucleic acid (Burgess, 2003; Zelnik, 2004; Singh *et al.,* 2012).

Accurate diagnosis of MD is necessary in other to differentiate the disease from other oncogenic diseases of birds like Avian Leukosis (ALV), Reticuloendothelosis Virus (REV) (Gopal *et al*., 2012). Confirmatory diagnosis of MD must be a combination of history, pathological diagnosis and positive identification of MD and not either of the two (Zelnik, 2004).

2.9.1Sample collection

One of the most important requirements for reliable diagnosis of MD which is most times overlooked is appropriate and proper collection of the infection material (Zelnik, 2004). For laboratory diagnosis non-coagulated whole blood can be used or Tumour from suspected birds. Feather follicle are also a very good source of MDV antigens and viral DNA for diagnosis(Davidson *et al.*,1986; Levy *et al.*,1991; Borenhastain *et al.*, 2002; Davidson *et al,* 2002; , Burgess, 2003; Zelnik, 2004, Gimeo *et al.,* 2005; Renz *etal.*,2006; Cortes *et al.*,2011).

2.9.2 Viral isolation

Blood is the most preferred sample for viral isolation although the virus can also be isolated from solid tumour like spleen, kidney or other tissues, cell free MDV can also can be isolated from feather tips (Zelnik, 2004; Wakenell *et al.*, 2008). For viral isolation the following cell culture are used: chicken embryo fibroblast (CEF), chicken kidney (CK) cells and Duck embryo fibroblast (DEF)(Burgess, 2004; Zelnik, 2004; Wakenell *et al.*, 2008). Primary isolation of field MDV, DEF and CK cells are preferred, while CEF is used for isolation of Serotype 2 and HVT(Witter and Schat, 2003; Burgess, 2004;Schat, 2005; Wakenell *et al.*, 2008,). Usually typical cytopathic effect (CPE) of MDV will develop in cell culture after 5-14 Days and morphologically differences between the three serotypes to be used as a serotype diagnosis(Burgess, 2004). Another method to differentiate MDV serotypes in cell culture is by staining the CPE bearing the serotypes with serotype –specific monoclonal antibodies using indirect immunofluorescence test (Wakenell *et al.*, 2008). Viral isolation can also be done by inoculation of suspected test material into the yolk sac of 4-day old embryonated chicken eggs or intravenously in 10-11 day old embryos, although cell culture is still the preferred method of viral isolation (Wakenell *et al.*, 2008; Singh *et al.*, 2012).

2.9.3Chicken inoculation

Viral isolation can also be done by inoculation of 1-5 day old chicks lacking maternally derived antibodies to MDV or HVT. Chicken that die should be necropsied and gross lesions examined (Wakenell *et al.*, 2008)

2.9.4 Serological methods

Several serological tests can be done to detect antibodies against MDV in Chickens although these are most useful in evaluating birds with no prior exposure to MD

vaccines of field strains (Zelnik, 2004; Wakenell *et al.,* 2008; Singh *et al.,* 2012). The serologic test include Agar gel precipitation test (AGPT), Fluorescent Antibody, Enzyme-linked immunosorbent assay (ELISA), virus neutralization test (Zelnik, 2004; Wakenell *et al.,* 2008, Singh *et al.,* 2012).

2.9.4.1Agar gel precipitation test

Using Agar gel precipitation test (AGPT),the serum is reacted with MD antigen in an agar medium. It is a simple test and the antigen suspension or even tips obtained directly from small feathers are placed in adjacent wells and the samples are left for 24-72 hours in a humidified atmosphere, a positive reaction can be identified by precipitates in the agar between wells (Sharma *et al.,* 1985;Zelnik, 2004; Wakenell *et al.,* 2008). Some research findings have also shown that AGPT can be used as a simple means of differentiation between virulent and attenuated /vaccines MDV strains (Zelnik, 2004).

2.9.4.2 Virus neutralization test

This test is used to detect neutralizing antibody in the serum or plasma of infected chicken. The test can be conducted with cell free virus suspension in SPGA-EDTA buffer with a titer of about (Wakenell *et al.,*2008).

2.9.5 Virus detection by molecular techniques

These are the most frequently used technique in the diagnosis of MDV in poultry. The molecular technique include conventional PCR, Nested PCR, Multiplex PCR, Nested PCR, Real time PCR, Quantitative Real time PCR and LAMP (Becker *et al.,*1992; Davidson *et al.,*2002; Burgess, 2003; Renz *et al.,* 2006; Angamuthu*et al.,*2012; Singh *et*

al., 2012; Xiuying *et al.,* 2012;Gopal *et al.,* 2012; Baigent *et al.,*2013; Wozniakowksi *et al.,*2013).

2.9.5.1 Conventional polymerase chain reaction

Polymerase Chain Reaction (PCR) was first developed in the 1983 by Kary Mullis which eventually won him the 1993 Nobel Prize for chemistry and the first PCR publication appeared in 1985 it has been used with great success in the diagnostics of bacterial and viral diseases for many years, in its various forms have cemented a place in modern pathology laboratory diagnosis (Becker *et al.,*1992;Burgess 2003; Zelnik, 2004; Pestana *et al.,*2010). The early 1990s was also the time when PCR methods were first introduced for the detection of poultry viruses such as chicken infectious anaemia virus, infectious bronchitis virus, infectious bursal disease virus, Marek's disease virus, infectious laryngotracheitis virus and avian leucosis viruses with more emphasis on detecting vaccine contaminants (Spackman,2012) The development of polymerase chain reaction (PCR) has revolutionized the field of molecular biology. The technique consists basically of the enzymatic synthesis of millions of copies of a target DNA sequence (Saiki *et al.,* 1985). Using a thermostable DNA polymerase (Saiki *et al.,* 1988), and a succession of cycles that includes denaturation of the template DNA, hybridization of specific DNA primers to the template and extension of the primers, it is possible to generate multiple copies of the target region enzymatically (Rodriguez, 1997). Thus, PCR provides a method for obtaining large quantities of specific DNA sequences from small amounts of DNA, including degraded DNA samples (Rodriguez, 1997).The main advantage of PCR, its rapidity and DNA from crude biological materials can be analyzed (Zelnik, 2004). When compared with virus isolation and other

classic techniques, PCR is the method of choice for diagnosis of many poultry viruses including Marek's disease virus (Rodriguez, 1997). The full length of the genome sequence of MDV 1, MDV2 and MDV3 have sequenced hence it makes differentiation of infection from vaccination and also differentiation of MDV serotypes that may be found simultaneously in the same chicken easier (Becker *et al.*,1992; Burgess, 2003; Singh *et al.*, 2012; Renz *et al.*,2013). The technique is very sensitive and every precaution must be taken to avoid cross-contamination and false result (Zelnik, 2004). PCR can amplify DNA from Tumors, feather follicle epithelium and Poultry dust with very good results (Davidson *et al.*, 1995; Baigent *et al.*, 2005; Davidson *et al.*, 2009; Baigent *et al.*, 2013). This technique can also differentiate MDV from other oncogenic viruses of poultry (Davidson *et al.*, 2002; Silva *et al.*, 2007). The technique has been used for detection of MDV in avian species in Nigeria (Owoade *et al.*, 2008; Jwander *et al.*,2012).

2.9.5.2 Nested polymerase chain reaction

This technique involves detection of meq oncogene of MDV in the spleens cells, feathers tips and peripheral blood of infected chicken (Lee *et al.*, 2000; Murata *et al.*, 2000;Singh *et al.*, 2012). The PCR technique can also be used for typing genetic markers, genetic mutation screening, detecting point mutations, cDNA cloning, DNA templates for DNA sequencing and invitro mutagenesis(Pestana *et al*., 2010).

2.9.5.3 Multiplex polymerase chain reaction

Due to the difficult in differentiating mix infection of REV, ALV and MDV, which have been reported in several publication(Davidson *et al.,* 2002; Davidson *et al.*,2009; Gopal *et al.,* 2012; Buscaglia *et al.*,2013). This version of PCR was developed to detect single or multiple infections of oncogenic viruses in poultry hence achieve rapid differential diagnosis of this diseases. Usually tissue samples of the liver, lungs and kidneys can be used for diagnosis (Gopal *et al.,* 2012; Singh *et al.,* 2012).

2.9.5.4 Quantitative real time polymerase chain reaction

Is a technique used to quantify MDV genome copies for simultaneous detection and quantitation of viral load in clinical and infected tissue (Signh *et al.,* 2012). It is a specialized technique that needs the usage of expensive equipment, it can be used for differentiation and quantitation of different MDV serotypes in feathers, tissue and dust. In addition it is also used in the monitoring of MD in poultry flocks and monitoring of MD vaccination as well as immune response to MD vaccination(Yunis *et al.,* 2004;Baigent *et al.,* 2005; Renz *et al.,*2006; Butter *et al.,* 2007; Islam *et al.*,2007; Tan *et al.,* 2007; Abdul-careem *et al.,* 2008; Gimeno, 2008;Cortes *et al.,* 2011; Jarosinki *et al.,* 2012; Biggs *et al.,*2012; Singh *et al.,*2012; Islam *et al.,* 2013). In addition to enhanced sensitivity, the benefits of real-time PCR assays over conventional endpoint detection methods include their large dynamic range, a reduced risk of cross contamination, an ability to be scaled up for high throughput applications and the potential for accurate target quantification (Hoffmann *et al.,* 2009).

2.9.5.4 Loop-mediated isothermal amplification method

A relatively new molecular technique which was first reported in year 2000 and has been the focus of extensive research efforts (Notomi *et al.,* 2000; Angamuthu *et al.,* 2012; Mori *et al.,* 2013). Despite the success of recorded in the use of PCR in the diagnosis of various diseases of Man and animals including MD, a major drawback of this technique is the use of expensive equipment like thermo cycler and high level of personnel expertise which may not be available in the field (Notomi *et al.,*2000; Angamuthu *et al.,*2012). This technique does not require the use of thermal cyclers and the technique involves the use of four-six different primers that recognizes 6-8 defined region of the targeted DNA, the Bst DNA polymerase is used to DNA strands and Isothermal condition is achieve using water bath for amplification of the target DNA(Wozniakowki *et al.,* 2011; Angamuthu *et al.,* 2012;Xiuying *et al.,* 2012;Wozniakowki *et al.,* 2013).

The Sequence of the detected gene is separated into six fragments for specific for the designed primers. Forward inner primer (FIP) and backward inner primer are designed for the initial step of reaction. The sequences of FIP and BIP are designed as follows: FIP consists of F1c fragment, a thymine (TTTT) linker, and the F2 fragment complementary to F2c region in meq sequence. BIP consists of the B1c fragment complementary to B1 sequence, a TTTT linker and B2 fragment. The two outer primers are B3 and F3 and take part in displacement of the newly synthesized strands from FIP and BIP primers. FIP anneals to F2c region in the target sequence and initiates complementary strand synthesis. Next F3 outer primer, in a considerably lower concentration than FIP, anneals to F3c sequence and initiates strand displacement,

releasing a newly synthesized FIP-linked strand. FIP-linked complementary strand, form a looped structure at its 59 end. Additionally, a single-stranded DNA on the 39 end of this structure is acting as a template for BIP that facilitates new DNA strand synthesis with the subsequent displacement DNA of newly synthesized strands initiated by B3. This leads to the formation of a dumbbell structure, which is used in the following steps of complex stem-loop DNA formation by auto cycling DNA synthesis (Wozniakowki *et al.,* 2011; Wozniakowki *et al.,* 2013).

Loop isothermal mediated amplification procedure is a nucleic acid amplification method that synthesizes large amount of DNA in a short period of time and with high specificity, this allows for easy and rapid visualization of the amplified products without need for gel electrophoresis. The result can be obtained by 2 hours (Notomi *et al.,*2000; Wei *et al.*2012). The LAMP techniquehave been reported to be highly specific and sensitive, more sensitive than PCR, it is simple technique that can be carryout in the field and by small laboratories.

In the last decade DNA amplification using LAMP has been applied for detection of the following human pathogen: hepatitis B virus, human parvovirus, severe acute respiratory syndrome (SARS), Mycobacterium tuberculosis Malaria parasite, Legionnella Species, Trypanosoma Brucei (Hopkins *et al.,* 2013;Mori *et al.,* 2013; Polley *et al.,*2013). There are many published work on animal pathogen in which LAMP was used with great results. This pathogens include infectious bursal disease, reticuloendotheliosis, Pseudo rabies , peste des petits ruminants virus and Avian leucosis virus, bovine viral diarrhea and foot and mouth disease (Xu *et al.,*2009; Deng

et al., 2010; Li *et al.*,2010; Wang *et al.*, 2011; Angamuthu*et al.*, 2012; Fan *et al.*,2012; Yamazaki, *et al.*, 2013).

2.10 Differential Diagnoses

Diagnosis is primarily based on age, clinical signs, history, gross and microscopic lesions (Singh *et al.*,2012). The oncogenic viruses that are known to affect poultry, these are Marek's disease virus, three retroviruses which includes reticulotheliosis, Avian Leucosis/sarcoma viruses and Avian retrovirus that are responsible for the rare neoplastic disease of Turkey known as Iymphoproliferative disease(Payne *et al.*,2000; Davidson *et al.*,2009). The clinical signs of infection caused by oncogenic viruses overlap and are similar both grossly and microscopically (Davidson, 2009). There are having reports of mix infection of MDV with either ALV or REV and possibly all the viruses in the same bird or flock (Davidson *et al.*, 1998). In addition ALV have been reported to contaminate MD vaccines, MD and REV have been known to recombine when co-infecting the same bird(Zavala *et al.*, 2006; Wang *et al.*, 2011; Mays*et al.*, 2012; Buscagia, 2013) .

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Area

The study area was Jos which is the administrative capital of Plateau State and situated approximately on latitude 9.6^0N, 10. 2^0 N and longitude 8.5^0 E, 9.1^0E The city lies close to the geographical centre of Nigeria with a population of about 816,824 residents, with an altitude of 1,217m above sea level. Its enjoys a more temperate climate than the rest of Nigeria (average monthly temperatures of 21°C - 25°C) from mid-November to late January, night temperatures drop as low as 11°C resulting in chilly nights. Jos is linked by road, rail and air to the rest of the country. Jos falls largely within the northern guinea savannah zone which consists mainly of short trees, grasses and the Plateau type of mosaic vegetation. Near some villages are thick hedges of cacti, which have been planted around household farms or compound lands. The staple food of the natives of Jos is maize, guinea corn, Irish potato, cassava, yams and *Acha* (a kind of mustard seed crop). The major activities are mining, civil service and commercial activities (Ashagidigbi *et al.,* 2011).

3.2Retrospective Prevalence of Marek's Disease in Jos, Plateau State

3.2.1 Retrospective study

A seven year (2007-2013)retrospective study of the common diseases of poultry was carried out based on the records obtained from the Veterinary Hospital/Clinic in the study area to ascertain prevalence of MD. The records were obtained from the following Veterinary Clinics or Diagnostic Centre:

1. Central Diagnostic Laboratory, National Veterinary Research Institute, Vom.
2. Evangelical Church Winning All (ECWA) Veterinary Clinic Bukuru, Jos.
3. Nannag Agrovet Services, Zaramangada, Jos.
4. Medical And Agricultural Services (MAAS) Veterinary Clinic, Niger Avenue, Jos.
5. Prime Veterinary Clinic, Old Railway line, Jos.
6. Unity Veterinary Clinic PIPC Shopping Complex, State LowCost Junction, Jos.

A case was defined as a farm that reported an outbreak of a disease and diagnosed based on history, clinical findings, and post- mortem findings (Wakawa *et al.*, 2012). The date, age, flock size, breed, species, type of birds, clinical signs and post- mortem findings of the diseases diagnosed were extracted from the clinic case record books and the data were entered into Microsoft Excel spread sheet and used for data analysis. Cases whose status was not known based on these criteria were considered as unknown or others.

The age was categorized as 0-6 weeks (chicks), >6-18weeks (growers),above18 weeks (layers) and not specified, i.e., cases that ages were not recorded. According to species, the data were categorized as chickens, turkeys and quails. By breed the data were categorized as local or improved breed, and type of bird will be broiler, breeder and layers. The seasons will be categorized into Dry (Jan-March), Pre-rainy (April-June),Rainy (July-Sept) andPre-dry (Oct-Dec) seasons.

3.3 Detection of Marek's Disease Virus Strains by Polymerase Chain Reaction and Loop-Mediated Isothermal Amplification Reaction

3.3.1Samples and sample collection

3.3.1.1 Sample Size

The number of samples collected was 157 samples and the samples were collected from April 2013-August 2014.

3.3.1.2 Sample collection

Samples were collected from clinically diagnosed cases of MD fromVeterinary Clinics/Hospitals in the study area.Tumoroussamples were collected during postmortem examinations from clinically diagnosed cases of MD from liver, spleen lungs, ovaries, heart, intestine, skin and feathers.

During postmortem examinations, visceral organs and skin showingtumorous gross lesions were taken aseptically and placed in properly labelled sample bottles.For each of the samples collected the clinico-pathological findings were properly documented in predesigned information form with respect to case number, detailed history, post-mortem findings and type of sample taken (Appendix 1).

Feathers samples were collectedfrom wings, neck and thighs of each bird at post-mortem. All samples wereplaced in samples bottles that are appropriately labeled and transported in flask with cold chain maintained and stored at -80^{0}C at the Avian Disease Research Laboratory, National Veterinary Research Institute, Vom.

3.3.1.3 Investigation of Marek's disease outbreaks

Farms with suspected outbreaks were investigated through farm visit and during the farm visit a complete history were taken including age, type of bird, source of birds, population, date of onset, management system, vaccination history, morbidity rate, mortality rate, egg production history. Post-mortem examination was done on dead birds; tissues and feather samples were collected.

3.4 Extraction Kit

Extraction of DNA was done using commercial kits, Qiaamp DNA mini kit (Qiagen, Germany).

3.5 Loop Isothermal Mediated Amplification ProcedureReaction Kit

OmniAmp™ RNA & DNA LAMP Kit (200 reactions) was procuredfrom Lucigen Corporation, U.S.A. for LAMP molecular technique andmanufacturer'sinstructions were followed.

3. 6 Loop Isothermal Mediated Amplification Procedure and Conventional Polymerase Chain Reaction Primers

The LAMP primers sequences usedwere designed as presented by Woźniakowski *et al.,* (2013). The conventional PCR primers sequences used were as presented by Renz *et al.* (2013). The primers were synthesized by Inqaba Biotechnical Industries, South Africa.

Table 3.1:Primer sequences for Loop-Mediated Isothermal Amplification Procedure

Test	Primer	Sequence	Marek's disease virus strain	Size
LAMP	MDV-1F3	TTCCCTCTTCTGCCCTCC	Serotype 1(RB-1B strain)	18bp
	MDV-1B3	TCCTGTTCGGGATCCTCG	Serotype 1(RB-1B strain)	
	MDV-1FIP	GTAAACCGTCCCCGGCGATGTTTT-GGGCATCTTCCCTGCATTG	Serotype 1(RB-1B strain)	
	MDV-1BIP	CTTTGTCCTGTTGGCCAGGCTCTTTT-GACGAGCATAAAGCCTCTCC	Serotype 1(RB-1B strain)	
	MDV-1LF	TACACGGCTCGGTAACAGGA	Serotype 1(RB-1B strain)	
	MDV-1LB	CCACATCCGGCTCCGGAGCC		
LAMP	MDV-3F3	ATAAATTATATCGCTAGGACAGAC	Serotype 3 (FC 126 Strain)	18bp
	MDV-3B3	ACGATGTGCTGTCGTCTA	Serotype 3 (FC 126 Strain)	
	MDV-3FIP	CCAGGGTATGCATATTCCATAACAGTTTTCCAAACGACCTTTATCCCA	Serotype 3 (FC 126 Strain)	43bp
	MDV-3BIP	CCAGAAATTGCACGCACGAGTTTTAGAATTTGTGCATTTAGCCTT	Serotype 3 (FC 126 Strain)	46bp
	MDV-3LF	TTGAGAAGAGGATCTGACTG	Serotype 3 (FC 126 Strain)	
	MDV-3LB	GCGTCATTGGTTTTACATTT	Serotype 3 (FC 126 Strain)	

F3: forward outer primer, FIP: forward inner primer, B3: backward outer primer, BIP: backward inner primers, LP: loop primers, LF= Loop Forward primers

Table 3.2 : Primer sequences for Polymerase chain reaction

Test	Primer	Sequence	Marek's disease virus strain	Product size
PCR				
	MDV-1F3	TTCCCTCTTCTGCCCTCC(forward)	Serotype 1(RB-1B strain)	200bp
	MDV-1B3	TCCTGTTCGGGATCCTCG(reverse)	Serotype 1(RB-1B strain)	
PCR	MDV-3F3	ATAAATTATATCGCTAGGACAGAC (forward)	Serotype 3 (FC 126 strain)	200bp
	MDV-3B3	ACGATGTGCTGTCGTCTA(reverse)	Serotype 3 (FC 126 strain)	
PCR	5'	5-TCGGAGAAGACGCAGGAA-3 (forward)	Serotype 1(Rispens strain)	152bp
	3'	5-GCTCATGACAAGCCAACTGTA-3 (reverse)	Serotype 1(Rispens strain)	
	5'	5_-TCGGAGAAGACGCAGGTC-3 (forward)	Serotype 1(Australian pathogenic strain)	202bp
	3'	5_-GTAAGCAGTCCAAGGGTCACC-3 (reverse)	Serotype 1(Australian pathogenic strain)	

F3:forward outer primer, FIP: forward inner primer, B3: backward outer primer, MDV- 1 =Marek's disease virus serotype 1 MDV- 3= Marek's disease virus serotype 3

3.7 Extraction of Marek's Disease Virus Deoxyribonuclelic Acid

Marek's disease virus DNA wasextracted using DNA mini kit (Qiagen Germany) following the manufacturer's instructions. Briefly,

1. One gram of tumorous tissue was weighted and grinded using pestle and mortar 1ml PBS was added, homogenate was placed in 1.5ml microcentrifuge tube, 20µl of proteinase K was added, mixing was done by vortexing and incubated at 56^0C on Stuart Scientific Block Heater for 1-3hours until the tissue was completely lysed, during this period vortexing was done 2-3 times per hour during the incubation period.
2. Briefcentrifuging was done in the 1.5ml microcentrifuge tube using an Eppendorf centrifuge 5417R to remove drops from the inside of the lid.
3. Addition of 200µl of buffer AL to the sample was done, mix by pulse-vortexing for 15seconds using Maxi mix II Thermolyne and incubation at 70^0C for 10 min using Stuart Scientific Block Heater was done by briefly centrifuging in the 1.5ml microcentrifuge tube in a Eppendorf centrifuge 5417R to remove drops from the inside of the lid
4. After the above step, 200 µl of absolute ethanol was added to the sample and vortex for 15s. After mixing briefly centrifuge the 1.5ml microcentrifuge tube in a Eppendorf centrifuge 5417R to remove drops from the inside of the lid.
5. The QIAamp mini spin column was carefully applied into a 2ml collection tube without wetting the rim. The lid wasclosed and centrifuged at 8000 rpm for 1min using Eppendorf centrifuge 5417R. The QIAamp mini spin column was placed in a clean collection tube provided in the kit and the tube containing the filtrate discarded.

6. The QIAamp mini spin column was opened and 500µl buffer AW1 added without wetting the rim. The cap closed and centrifuged at 8000rpm for 1min using Eppendorf centrifuge 5417R. The QIAamp mini spin column was placed in a clean collection tube provided in the kit and the tube containing the filtrate discarded.
7. The QIAamp mini spin column was opened and 500 µl buffer AW2 added without wetting the rim. Close cap and centrifuge at 14,000rpm for 3min using Eppendorf centrifuge 5417R.
8. The QIAamp mini spin column was placed in a clean collection tube provided in the kit and discard the tube containing the filtrate. Cap was closed and centrifuged at 14,000rpm for 1min using Eppendorf centrifuge 5417R, to eliminate the chance of possible buffer AW2 carryover.
9. The QIAamp mini spin column was placed in a clean collection tube and the tube containing the filtrate discarded. The QIAamp mini spin column was opened and 100µl buffer AE added. Incubation was done at room temperature for 1min and centrifuged at 8000rpm for 1min.
10. The QIAamp mini spin column was discarded and the DNA collected in the collection tube stored at 20^0C.

The following were the MDV positive controls used for LAMP and PCR:

1. Biovac 107 Live Marek's disease vaccine HVT-FC 126 Strain produced by Biovac Ltd P.O.B 300 Akiva 30600, Israel.
2. Cryomarex frozen Marek's disease vaccine Rispens strain produced by Merial SAS France.
3. Marek's disease virus serotype 1was from NVRI Vom

3.8 Polymerase Chain Reaction

The conventional PCR was done as described by Woźniakowski *et al.*(2011) and according to manufacturer's instructions using Thermo Scientific Dream Taq Green PCR master mix (2X) and after optimizing with the kit. The Protocol is as follows:

	1X
DreamTaq Green DNA Polymerase mastermix	12.5µl
MD B_3 (Primer)	2µl
MD F_3 (Primer)	2µl
Nuclease free water	6.5µl
MDV DNA	2 µl
Total	25 µl

3.8.1 Thermocycler protocol

The PCR mixes and the thermal cycles used were optimized in National Veterinary Research Institute Vom. The PCR mix was initially vortexedand 23 µl was dispensedinto each PCR tube, 2 µl of the MD DNA was added to the PCR mix and was vortexed and placed in the thermocycler to undergo the PCR thermal procedure as follows:

94 ^{0}C for 3 minutes	Initial denaturation	
94 ^{0}C for 30 seconds	Denaturation	35 cycles
55 ^{0}C for 30 seconds	Annealing	
72 ^{0}C for 1 minute	Extension	
72 ^{0}C for 7 minutes	Final extension	

The annealing temperature was varied(52^0C was used for the HVT primers, 55^0C was used for the conventional PCR reaction) depending on the type of primers used according to the manufacturer's instructions. The thermocycling procedure was done using GeneAmp PCR System 9700 by Applied Biosystem.

3.8.2 Electrophoresis of the polymerase chain reaction product

3.8.2.1 Gel preparation

a. 1.5g of agarose powder was weighted
b. 100 ml of 1X TBE buffer was added and agitate
c. Boil in a microwave oven at Maximum heat for 3minutes
d. Solution was cooled to about 45^0C under Tap water or swirl gently at room temperature.
e. 5 µl of ethidium bromide was added
f. Gel was poured out into gel casting tray with attached combs, placed at room temperature for polymerization to take place (15mins).

The first and last well of each lane of the gel were loaded with 5 µl of ladder or maker. From each sample, 10 µlwere loaded into subsequence wells using single channel micropipette, using master mix there was no need of the loading dye because they were already incorporated into the wells. The electrophoresis tank was then connected to the power source and then run the gel for 20-30 minutes at 200V. DNA migrates from cathode (-ve) to anode (+ve). The bands formed were made visible using UV light connected to a camera (photographed) and then using a connected computer reader.

3.9 Loop –Mediated Isothermal Amplification Procedure

Loop –mediated isothermal amplification procedure was carried out as described by Woźniakowski *et al.*,(2013) using Omniamp RNA and DNA LAMP kit (Lucigen U.S.A.).

3.9.1Optimization of Loop –Mediated Isothermal Amplification Procedure.

To validate the LAMP kit, the initial optimization was done using the positive control provide in the LAMP kit

The 2nd stage of the optimization of LAMP was done by varying the different concentration of the following:

1. The Magnesium chloride
2. The dNTPs
3. Betaine
4. Nuclease free water
5. The forward inner primers
6. The backward inner primers
7. Forward primer
8. The backward primer
9. The loop forward primer
10. The loop backward primer
11. The DNA template

The time and temperature of the reaction was also varied to obtain the best results.

The variations of the reagents stated above were done as follows:

Component	Vol in µl			
	1X	1X	1X	1X
Nuclease-free Water	11.00	6.00	5.00	8.65
10X DNA Polymerase Buffer C	2.5	2.5	2.5	2.5
OmniAmp DNA Polymerase, 50X	1.00	1.00	1.00	0.5
F3 (10pmol)	1.00	1.00	1.00	0.375
B3 (10pmol)	1.00	1.00	1.00	0.375
LF (20pmol)	1.00	1.00	1.00	0.75
LB (20pmol)	1.00	1.00	1.00	0.75
100 mM MgSO4	0.00	1.00	3.00	2.0
FIP (40pmol)	1.00	1.00	1.00	1.5
BIP (40pmol)	1.00	1.00	1.00	1.5
dNTPs (25 mM each)	1.00	2.00	4.00	1.6
Betaine	1.00	4.00	2.00	2.5
DNA template	2.5	2.5	1.50	2
Total (vol. in µl)	25.00	25.00	25.00	25.00

The following was the optimized value for the LAMP reagents use for the LAMP reaction:

Component	Volume in µl
	1X
Nuclease-free Water	5.00
10X DNA Polymerase Buffer C	2.5
OmniAmp DNA Polymerase, 50X	1.00
F3 (10pmol)	1.00
B3 (10pmol)	1.00
LF (20pmol)	1.00
LB (20pmol)	1.00
100 mM MgSO4	3.0
FIP (40pmol)	1.00
BIP(40pmol)	1.00
dNTPs (25 mM each)	4.00
Betaine	2.00
Total	23.50

The LAMP reaction was carried out in 1.5ml microcentrifuge eppendorf tube as follows:

1. Thawed kit components were held on ice.
2. All components were mixed well before use by vortexing
3. The reaction mix was done as shown below according to the Manufacturer's recommendation. All the components were held on ice to prevent background activity of enzyme.

4. Once all reagents have been added, the reaction was mixed completely. This step is required to ensure uniform distribution of all reaction components.
5. 1.5 µl of MDV DNA was added
6. Cap tubes were vortexedbriefly to collect prior to incubation.
7. Incubate at 70°C for 30 minutes.
8. Amplification reactions was stop immediately by holding the mix on ice at 4°C to stop enzyme activity
9. Second stage incubation was done at 95 °C for 5 minutes.

The first lane of the gel was loaded with 5 µl of ladder or maker. From each sample, 10 µl are loaded into subsequence wells using single channel micropipette, using master mix there is no need of the loading dye because they are incorporated into the wells. The electrophoresis tank was then connected to the power source and then run the gel for 20-30 minutes at 200V. DNA migrates from cathode (-ve) to anode (+ve). The gel was placed on a UV transilluminator and viewed on a computer monitor through a digital camera attached to a hood on the transilluminator.

CHAPTER FOUR

RESULTS

4.1 Results of Retrospective Study

4.1.1 Yearly distribution of Marek's' disease diagnosed in Veterinary Clinics and a Diagnostic Centres in Jos, Plateau State (2007-2013).

The total number of case recorded over a 7 years period was 28,723 out of which 571(1.99%) were diagnosed as MD. The year 2013 had the highest prevalence of 8.48% for MD and 2011 had the lowest prevalence rate of 0.42% (Table 4.1).Total number of cases diagnosed as tumour were 3,151(10.99%)(Appendix 2).

4.1.2Monthly distribution of Marek's' disease cases diagnosed in Veterinary Clinics and a Diagnostic Centres in Jos, Plateau State (2007-2013)

The prevalence of Marek's disease was 1.99%. The month of March had the highest prevalence of 2.96% for MD and the least monthly prevalence was December (Table 4.2) The odds ratios of the months of May to October and December at 95% confidence interval (CI) was significant for MD. Marek's disease cases were recorded throughout the year. Cases diagnosed as tumour were also recorded throughout the year and the highest prevalence was March (13.82%) similar to MD (Appendix 3).

4.1.3 Seasonal distribution of Marek's disease cases diagnosed in Veterinary Clinics and a Diagnostic Centrein Jos, Plateau State (2007-2013)

Highest MD prevalence of 2.48% was recorded in the dry season (Jan-March) and the lowest was in the rainy season(July-Sept) for the MD with prevalence rate of 1.74%. The odds ratio was significant for all seasons of the year (Table 4.3). Similarly the

highest prevalence of cases diagnosed as tumour was during the dry season (14.2%)(Appendix 4).

4.1.4 Age distribution of Marek's disease cases diagnosed in Veterinary Clinics anda Diagnostic Centre in Jos, Plateau State (2007-2013)

Poultry of the age group of 18 weeks and abovehad the highest prevalence of 8.76% for MD, while group whose age was not-specified (81.79%) (Table4.4). Furthermore MD was diagnosed in exotic layer chicken of all age groups from the records obtained. Cases diagnosed as tumour had the highest prevalence in poultry above 18 weeks of age (Appendices 4 and7).

4.1.5 Distribution of Marek'sdisease in different type and specie of poultry in Jos, Plateau State (2007-2013)

From the records obtained the prevalence of MD in different type and specie of poultry was as follows: broilers (4.73%), layers (46.72%) and turkeys (0.72%). Not specified type or specie of poultry had prevalence of 48.69% .

Table 4.1: Yearly distribution of Marek's disease diagnosed in poultry from veterinary clinics and a diagnostic centre in Jos, Plateau State(2007-2013)

Year	Marek's disease cases (%)	Non Marek's disease cases	Total cases	Odds ratio	Confidence interval	p-value
2007	16 (0.83)	1912	1928	1		
2008	122 (2.94)	4028	4150	3.62	2.13-6.11	0.0001
2009	76 (1.03)	7332	7408	1.24	0.72-2.28	0.447
2010	132 (2.66)	4834	4966	3.26	1.94-5.50	0.0002
2011	26 (0.42)	6207	6233	0.50	0.27-0.94	0.0363
2012	122 (3.90)	3008	3130	4.84	2.86-8.18	0.0001
2013	77 (8.48)	831	908	11.07	6.42-19.09	0.0001
Total	**571 (1.99)**	**28,152**	**28,723**			

Table4.2: Monthly distribution of Marek's disease diagnosed in poultry from Veterinary Clinics and a Diagnostic Centre in Jos, Plateau State(2007-2013)

Year	Marek's disease cases (%)	Non Marek's disease cases	Total cases	Odds ratio	Confidence interval	p-value
January	31 (1.92%)	1581	1612	1		
February	46 (2.28%)	1968	2014	1.12	0.73-1.89	0.453
March	81 (2.96%)	2652	2733	1.56	1.03- 2.36	0.037
April	86 (2.91%)	2869	2955	1.65	1.09-2.59	0.017
May	30 (0.90%)	3321	3351	0.46	0.27-0.76	0.003
June	61 (1.87%)	3200	3261	0.97	0.61-1.56	0.899
July	49 (1.86%)	2583	2632	0.96	0.61-1.52	0.886
August	46 (1.72%)	2633	2679	0.89	0.56-1.41	0.620
September	33 (1.61%)	2017	2050	0.83	0.51-1.37	0.470
October	40 (1.91%)	2059	2099	0.97	0.60-1.57	0.960
November	51 (2.58%)	1927	1978	1.35	0.84-2.12	0.191
December	17 (1.25%)	1342	1359	0.64	0.35-1.17	0.147
Total	**571 (1.99%)**	**28,152**	**28,723**			

Table 4.3: Seasonal distribution of Marek's disease in poultry from Veterinary Clinics and Diagnostic Centre in Jos, Plateau State (2007-2013).

Season	Marek's disease cases (%)	Non Marek's disease cases	Total Cases	Odds ratio	Confidence interval	p-value
Dry (Jan-March)	158 (2.48)	6201	6359	1		
Pre rainy (April-June)	177 (1.85)	9390	9567	0.73	0.59-0.91	0.006
Rainy (July-Sept)	128 (1.74)	7233	7361	0.68	0.53-0.86	0.001
Pre dry (Oct- Dec)	108 (1.99)	5328	5436	0.78	0.61-0.99	0.048
Total	**571(1.99)**	**28,152**	**28,723**			

Table 4.4: Age distribution of Marek's disease diagnosed in different species of poultry from veterinary clinics and a diagnostic centre in Jos, Plateau State (2007-2013).

Age group in weeks	Broiler(%)	Layer(%)	Turkey(%)	Species not specified (%)	Total(%)
0-6	0	15(2.63)	0	0	15(2.63)
>6-18	0	37(6.48)	2(0.35)	0	39(6.83)
18>	0	50(8.76)	0	0	50(8.76)
Not specified	27(4.73)	162(28.3)	0	278	467(81.79)
Total	**27(4.73)**	**264(46.23)**	**2(0.72)**	**278(48.69)**	**571(100)**

4.1.6 Distribution of concurrent infection of Marek's' disease and other diseases diagnosed in Veterinary Clinics and Diagnostic centre in Jos, Plateau State

Marek's disease was diagnosed concurrently with seven different diseases (Table 4.5). Marek's disease was diagnosed with coccidiosis (6.65%), colibacillosis (4.20%), salmonellosis (0.18%), Newcastle disease (8.41%), fatty liver syndrome (0.18%) and fowl typhoid (1.05%). In layers, MD was concurrently diagnosed with coccidiosis (13.64%) colibacillosis (8.33%), Newcastle disease (14.77), fatty liver syndrome (0.38%) and fowl typhoid (2.27). In broilers, Marek's disease was diagnosed with salmonellosis (3.70%), Newcastle disease (33.33%) and coccidiosis (7.41%).

4.2 Detection of different serotypes and strainsof Marek's disease virus DNA in different samples from poultry by Polymerase Chain Reaction

Marek's disease virus serotype 1 (RB-1B) was detected in feathers (90.38%), spleen (81.33%), heart (100%), lungs (100%), kidney (100%) and skin (100%) by PCR. The Rispens (MDV1) strain used for vaccines was detected in feathers (89.36%), spleen (78.95%) and liver (100%). The MDV pathogenic (Australian strain) was also detected in the feathers (5.88%) and spleen (11.54%). The Herpes virus of Turkey (FC 126) was detected in feather (3.13%), liver (100%) and spleen (14.29%) (Table 4.6). The samples are positive at 200 base pairs (bps) for MDV1 (RB-1B) 152 bps for Rispens, and 202bps for pathogenic MDV 1 and HVT, (Plates I, II, VI, VII and VIII).

Table 4.5: Distribution of concurrent infection of Marek's disease and other diseases diagnosed in poultry from Veterinary Clinics and a Diagnostic centre in Jos, Plateau State (2007-2013)

Disease	Layers(%)	Broilers(%)	Turkeys(%)	Type and species not specified(%)	Total
Marek's disease alone	156(59.09)	15(55.56)	2(100)	276(99.28)	449(78.63)
Marek's disease + Salmonellosis	0	1(3.70)		0	1(0.18)
Marek's disease + Colibacillosis	22(8.33)	0		2(0.72)	24(4.20)
Marek's disease + Newcastle disease	39(14.77)	9(33.33)		0	48(8.41)
Marek's disease + Coccidiosis	36(13.64)	2(7.41)		0	38(6.65)
Marek's disease +Respiratory tract infection + Colibacillosis	1(0.38)	0		0	1(0.18)
Marek's disease + Fatty liver syndrome	1(0.38)	0		0	1(0.18)
Marek's disease + Coccidiosis + Newcastle disease	3(1.14)	0		0	3(0.53)
Marek's disease + Fowl typhoid	6(2.27)	0		0	6(1.05)
Overall prevalence	**264(46.23)**	**27(4.73)**	**2(0.35)**	**278(48.69)**	**571(100)**

Table 4.6:Detection of different serotypes and strainsof Marek's disease virus in different samples of poultry in Jos, Plateau State by polymerase chain reaction

Type of sample	Marek's disease virus RB-1B strain (serotype 1)			Marek disease virus Rispens strain (serotype 1)			Marek's disease virus Pathogenic Australian strain (serotype 1)			Herpes Virus of Turkey (serotype 3)		
	No. of samples tested	No of samples positive	% of samples positive	No. of sample tested	No of samples positive	% of samples positive	No. of sample tested	No of samples positive	% of samples positive	No. of sample tested	No of samples positive	% of samples positive
Feathers	52	47	90.38	47	42	89.36	34	2	5.88	32	1	3.13
Liver	24	24	100.00	4	4	100.00	2	0	0.00	1	1	100.00
Spleen	75	61	81.33	57	45	78.95	26	3	11.54	21	3	14.29
Heart	1	1	100.00	0	0	0.00	0	0	0.00	0	0	0.00
Lungs	1	1	100.00	0	0	0.00	0	0	0.00	0	0	0.00
Kidneys	1	1	100.00	0	0	0.00	0	0	0.00	0	0	0.00
Skin	3	3	100.00	1	1	100.00	1	0	0.00	0	0	0.00
Total	157	138	87.90	109	92	84.40	63	5	7.94	54	5	9.09

4.3 Detection of Different serotypes and strains of Marek's disease virus DNA in Different Type and Specie of Poultry by Polymerase Chain Reaction

The MDV Serotype 1 DNA (RB-1B) was detected in broilers, (88%), layers (84.68%), local chicken (75%) and quails (100%) (Table 4.6). For MDV1 Rispens vaccine strain was detected in broilers (86.6%), layers (83.52%)(Plate II), local chickens (50%) and quails (100%)(Table 4.7, Plates III and V)

4.4 Detection of Different serotypes andstrains of Marek's disease Virus DNA in the Spleen of different type and Specie of Poultry by Polymerase Chain Reaction

The following serotypes were detected from the spleen samples of broilers: MDV Serotype 1 (RB-1B) (72.73%) and MDV Rispens strain (71.43%). From the spleen samples of layers, MDV Serotype 1 (RB-1B) (86.1%) and MDV Rispens strain (80%), MDV pathogenic (Australian strain) (9.52%) and HVT (FC126) (13%) were detected by PCR. Only MDV1 (RB-1B) (66.67%) was detected by PCR from spleen samples of local chicken (Table 4.8, Plates 1and V)

4.5 Detection of Different Serotypes of Marek's Disease virus DNA in in the feathers of different type and specie of poultry by Polymerase chain reaction

The MDV Serotype 1 (MB-1B) was detected in the feathers of broilers (100%) and layers (85%) by PCR. While MDV Rispens strain was detected in the feathers of broilers (100%) and layers (86%) and MDV pathogenic strain was detected in the feather of layers (24%) by PCR (Table 4.9)

Table 4.7:Detection of the different serotypes and strain of Marek's disease virus in different type and specie of poultryin Jos, Plateau Stateby polymerase chain reaction.

Species	Marek's disease virus RB-1B strain (serotype 1)			Marek disease virus Rispens strain (serotype 1)			Marek's disease virus Pathogenic Australian strain (serotype 1)			Herpes Virus of Turkey (serotype 3)		
	No. of sample tested	No of samples positive	% of samples positive	No. of sample tested	No of samples positive	% of samples positive	No. of sample tested	No of samples positive	% of samples positive	No. of sample tested	No of samples positive	% of samples positive
Broiler	25	22	88.00	15	13	86.67	8	0	0.00	6	1	16.67
Layer	111	94	84.68	91	76	83.52	40	5	12.50	32	4	12.50
Local chicken	8	6	75.00	4	2	50.00	2	0	0.00	3	1	33.33
Quail	2	2	100.00	1	1	100.00	1	0	0.00	0	0	0.00
Total	146	124	84.93	111	92	82.88	51	5	9.80	41	6	14.63

Table 4.8: Detection of the different serotypes and strain of Marek's disease virus in spleen of different type and species of poultry in Jos, Plateau Stateby polymerase chain reaction.

Specie	Marek's disease virus RB-1B strain (serotype 1)			Marek disease virus Rispens strain (serotype 1)			Marek's disease virus Pathogenic Australian strain (serotype 1)			Herpes Virus of Turkey (serotype 3)		
	No. of sample tested	No of samples positive	% of samples positive	No. of sample tested	No of samples positive	% of samples positive	No. of sample tested	No of samples positive	% of samples positive	No. of sample tested	No of samples positive	% of samples positive
Broiler	11	8	72.73	7	5	71.43	3	0	0.00	0	0	0
Layer	58	50	86.21	50	40	80.00	21	2	9.52	24	3	13
Local	3	2	66.67	0	0	0.00	0	0	0.00	0	0	0
Quail	1	1	100.00	0	0	0.00	0	0	0.00	0	0	0
Total	73	61	83.56	57	45	78.95	24	2	8.33	24	3	13

Table 4.9:Detection of the different serotypes and strainsof Marek's disease virusin feathers of different type of poultry in Jos, Plateau State by polymerase chain reaction.

Specie	Marek's disease virus RB-1B strain (serotype 1)			Marek disease virus Rispens strain (serotype 1)			Marek's disease virus Pathogenic Australian strain (serotype 1)			Herpes Virus of Turkey (serotype 3)		
	No. of sample tested	No. of samples positive	% of samples positive	No. of sample tested	No. of samples positive	% of samples positive	No. of sample tested	No. of samples positive	% of samples positive	No. of sample tested	No. of samples positive	% of samples positive
Broiler	7	7	100	7	7	100	5	0	0	5	1	20
Layer	47	40	85	37	32	86	28	2	7	24	0	0
Local chicken	1	0	0									
Total	**55**	**47**	**85.45**	**44**	**39**	**88.64**	**33**	**2**	**6.06**	**29**	**1**	**3.45**

4.6 Detection of the Different Serotypes of Marek's Disease Virus in poultry of Different Ages by Polymerase Chain Reaction

Different serotypes of MDV were detected by PCR in the different age groups as shown in Table 4:10. For 0-6 weeks age the positive results is as follows: MDV1 100 %(4), Rispens 50%(2). The second age group is the >6-18 weeks old, 83.33% (10) of the samples were positive for MDV RB-1B strain (Plate XI) 90%(9), 0% and 25% (2) of the samples were positive for Rispens, Pathogenic and HVT serotypes respectively by PCR (Table 4:11).

For age group of above 18 weeks, in 86.41% (89) samples MDV1 was detected by PCR, Rispens strain was detected in 83.78% (62) samples by PCR, while the Pathogenic Australian strain was detected in 11.11% (5) samples analyzed by PCR. Furthermore 3.33% of the samples were positive for HVT serotype 3 by PCR.

4.7 Detection of serotype 3 Marek's Disease Virus using Loop Mediated Isothermal Amplification Procedure

Marek's disease serotype 3 was detected by LAMP in broilers, local chicken, and layers (see Table 4:11). The result also indicted that HVT was detected in the liver, feathers and skin samples by LAMP. The result shows multiple clear bands as a result of amplification of six different targeted segments of the virus (Plates IX and X).

4.8 Detection of Different Serotypes and Strains of Marek's Disease Virus by Polymerase Chain Reaction and Diagnosis of Marek's Disease using Histopathology

The results shows that MDV1RB-1B(85.7%), MDV Risepens strain (100%) was detected by PCR and MD was diagnosed using histopathology (100%) (Table 4.12, PlatesXVII and XVIII). In some samplesMDV was not deteected by PCR but MD was diagnosed by histopathology, this could as result of different the strains of MDV

Table 4.10: Detection of the different serotypes of Marek's disease virus in poultry of different ages in Jos, Plateau State by polymerase chain reaction.

Age group in weeks	Marek's disease virus RB-1B strain (serotype 1)			Marek disease virus Rispens strain (serotype 1)			Marek's disease virus Pathogenic Australian strain (serotype 1)			Herpes Virus of Turkey (serotype 3)		
	No. of sample tested	No of samples positive	% of samples positive	No. of sample tested	No of samples positive	% of samples positive	No. of sample tested	No of samples positive	% of samples positive	No. of sample tested	No of samples positive	% of samples positive
0-6	4	4	100.00	4	2	50.00	0	0	0.00	0	0	0.00
>6-18	12	10	83.33	10	9	90.00	9	0	0.00	8	2	25.00
18>	103	89	86.41	74	62	83.78	45	5	11.11	30	1	3.33
Total	119	103	86.55	88	73	82.95	54	5	9.26	38	3	7.89

Table 4.11: Detection of Marek's disease virus of serotype 3 using loop mediated isothermal amplification procedure in different samples of different type of poultry.

Type of chicken	Type of sample	Result
Broiler	Spleen	Negative
Broiler	Liver	Positive
Layer	Feather	Positive
Layer	Feather	Positive
Local chicken	Skin	Positive

Table 4.12: Detection of different serotypes Marek's disease virus by polymerase chain reaction and diagnosis of Marek's disease using histopathology in Jos, Plateau State.

S/No	Species	Gross pathology	Histopathology	Polymerase chain reaction		
				Marek's disease virus serotype 1 (RB-1B)	Marek's disease virus Rispens strain Serotype 1	Marek's disease virus Pathogenic strain Serotype 1
JNN 30	Broiler	Enlarged Spleen, enlarged liver with tumors, skin tumours	spleen (positive)	liver (positive)	liver (positive)	
JNN 214	Broiler		spleen (positive)	spleen (positive)	spleen (positive)	spleen (negative)
JNN 215	Broiler	Enlarged heart with tumors	heart (positive)	heart (positive)		
JNN 13	Layers	Enlarge liver and emaciation	spleen (positive)	liver (positive)		
JNN 201	Layers		liver (positive)	spleen (positive) feathers (positive)	spleen (positive) feathers (positive)	
JSS 209	Layers	Enlarge spleen and tumorous liver	liver (positive)	spleen (positive)	spleen (positive)	spleen (negative)
JSS 213	Layers	Enlarged spleen and liver with tumours	liver (positive)	spleen (positive)		
JSS 214	Layers		spleen (positive)	spleen (negative)		
JSS 215	Layers	Enlarged spleen and liver with tumorous lesions	spleen (positive)	spleen (positive) feathers (positive) liver (positive)	spleen (positive)	spleen (negative)
JNN 217	Layers	Enlarged liver with tumor like lesions	spleen (positive)	liver (positive)	liver (positive)	
JNN 223	Layers	Tumorous like lesions on the spleen	spleen (positive)	spleen (positive)		
JNN 224	Layers	Enlarged spleen and liver	spleen (positive)	spleen (negative) feathers (negative)		
JNN 225	Layers	Enlarged kidneys and spleen	kidneys (positive)	spleen (negative) feathers (negative)	spleen (positive)	spleen (positive)
JNN 207	Local chicken	Enlarged spleen	spleen (positive)	spleen (negative) feather (negative)		
JNN 208	Local chicken	Enlarged spleen	spleen (positive)	spleen (positive)		
JNN 209	Local chicken	Enlarged spleen	spleen (positive)	spleen (positive)		
JNN 221	Local chicken	Enlarged liver and tumorous lungs	lung (positive)	liver (positive), lungs (positive)		

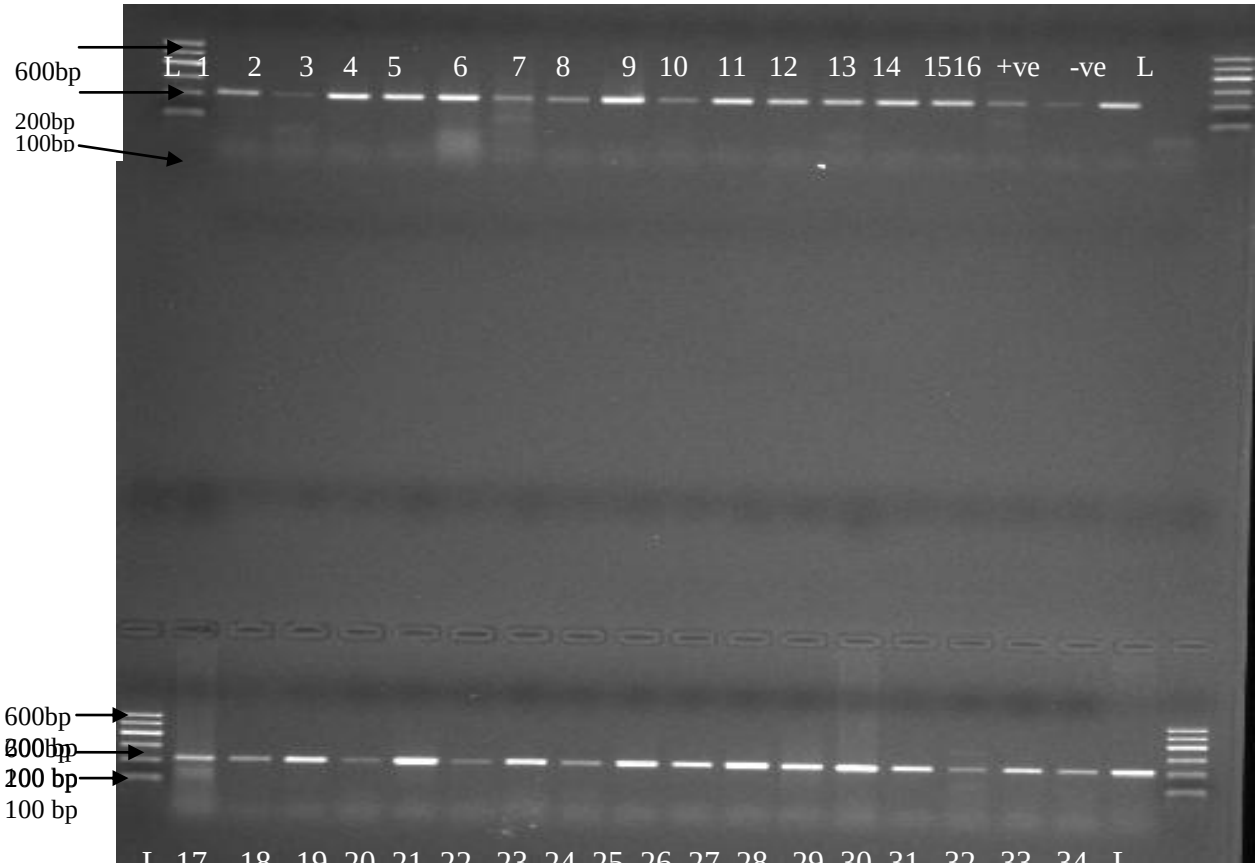


Plate I: Bands on 1% agarose gel of Polymerase chain reaction amplification product of Marek's disease virus Deoxyribonucleic acidfrom spleen samples using the Marek's disease virus serotpe 1 (RB-1B) specific primers, the positive band is at 200 bps -ve is the negative control, +ve is the positive control. L is the Ladderand 100bp. Numbers 1-34 are positive

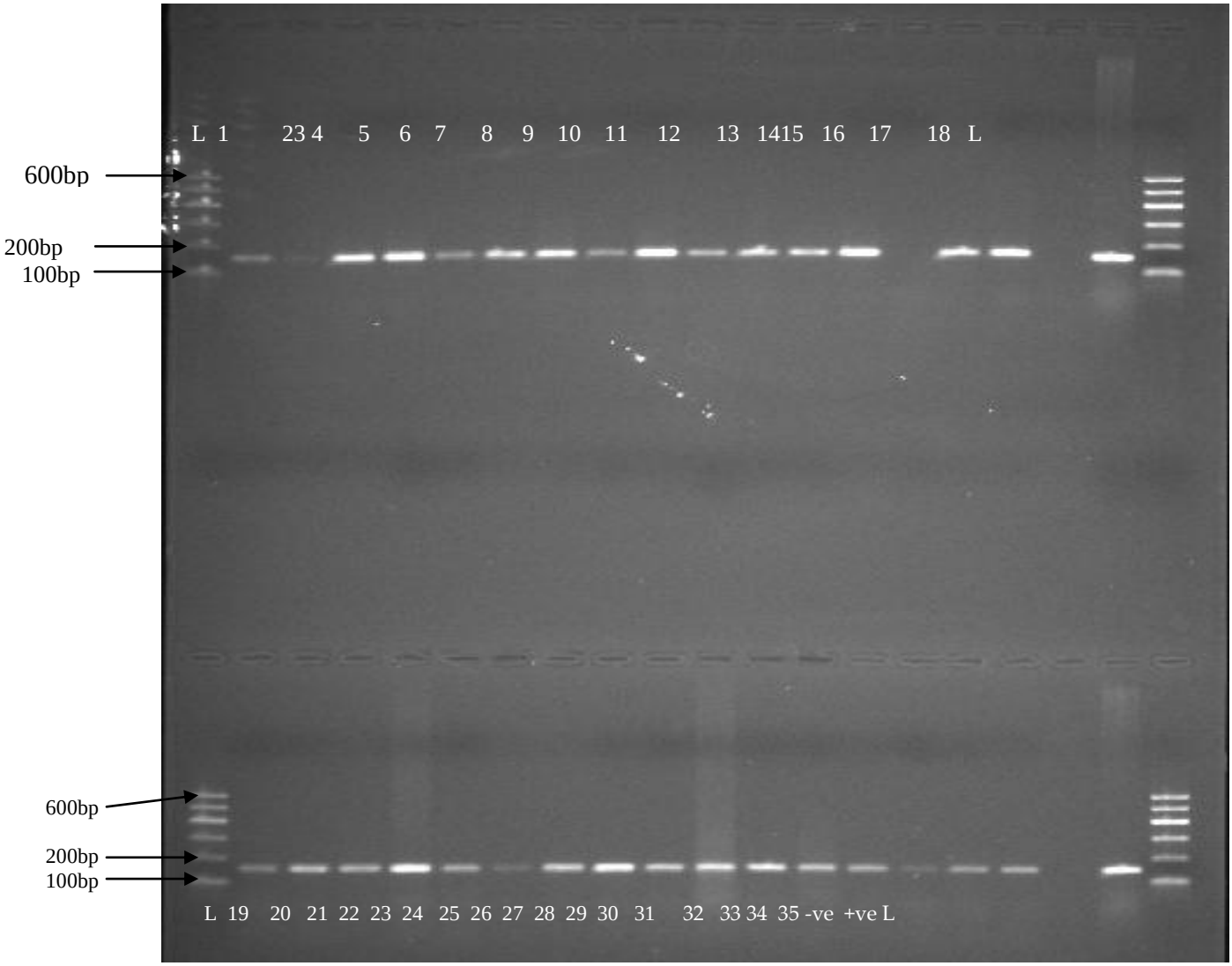


Plate II: Bands of 1% agarose gel of Polymerase chain reaction amplification products of Marek's disease virus Deoxyribonucleic acid from feather samples of layer using the Rispens specific primers, the positive band is at 152 bps -ve is the negative control, +ve is the positive control. The blanks wells are the negatives (14, 17). Positive are1-13, 15, 16, 18-35.

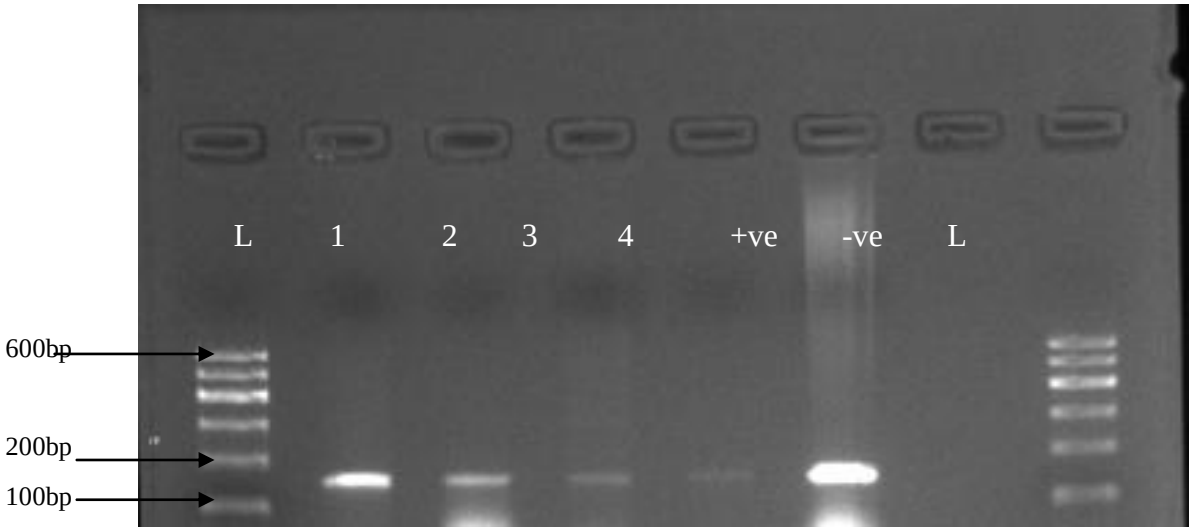


Plate III: Bands of1% agarose gel of Polymerase chain reaction amplification products of Marek's disease virus Deoxyribonucleic acid using the Marek's disease serotype 1 Rispens strain specific primers. The positive band is at 152 bps -ve is the negative control, +ve is the positive control. 1 is the liver of broiler, 2 is the liver of layer, 3 is the liver of local chicken and 4 is skin of Local chicken

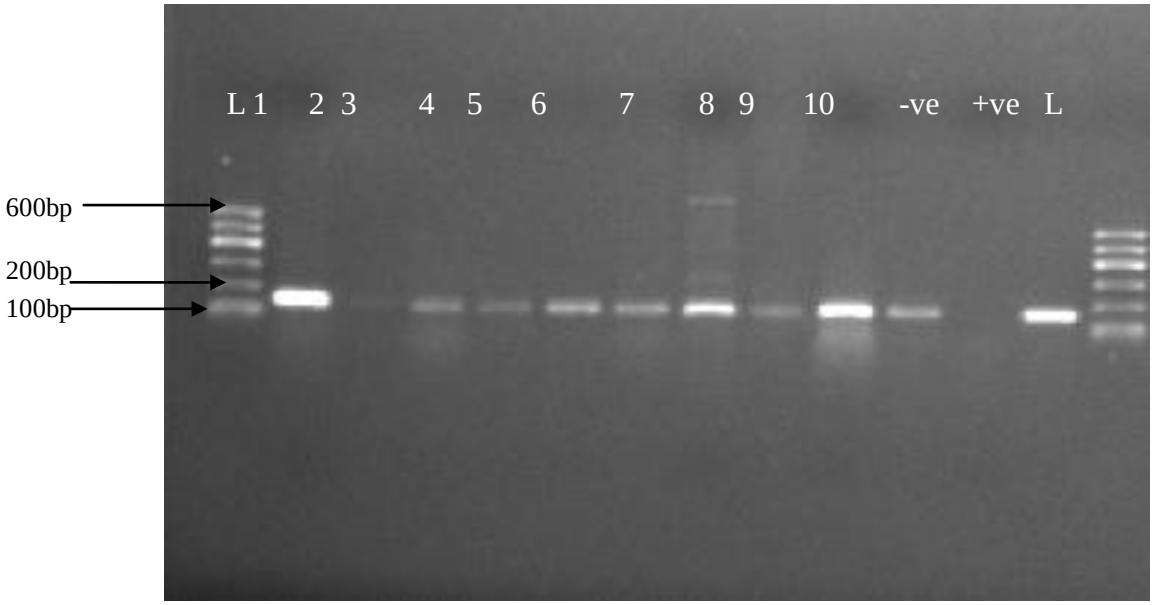


Plate IV:Bands of 1% agarose gel ofPolymerase chain reaction amplification product of Marek's disease virus Deoxyribonucleic acid from spleen samples using the Marek's disease virus serotype 1 Rispens specific primers. The positive band is at 152 bps -ve is the negative control, +ve is the positive control. Blank wells are the negative L is the ladder or marker which 100bp.1-10 are positive

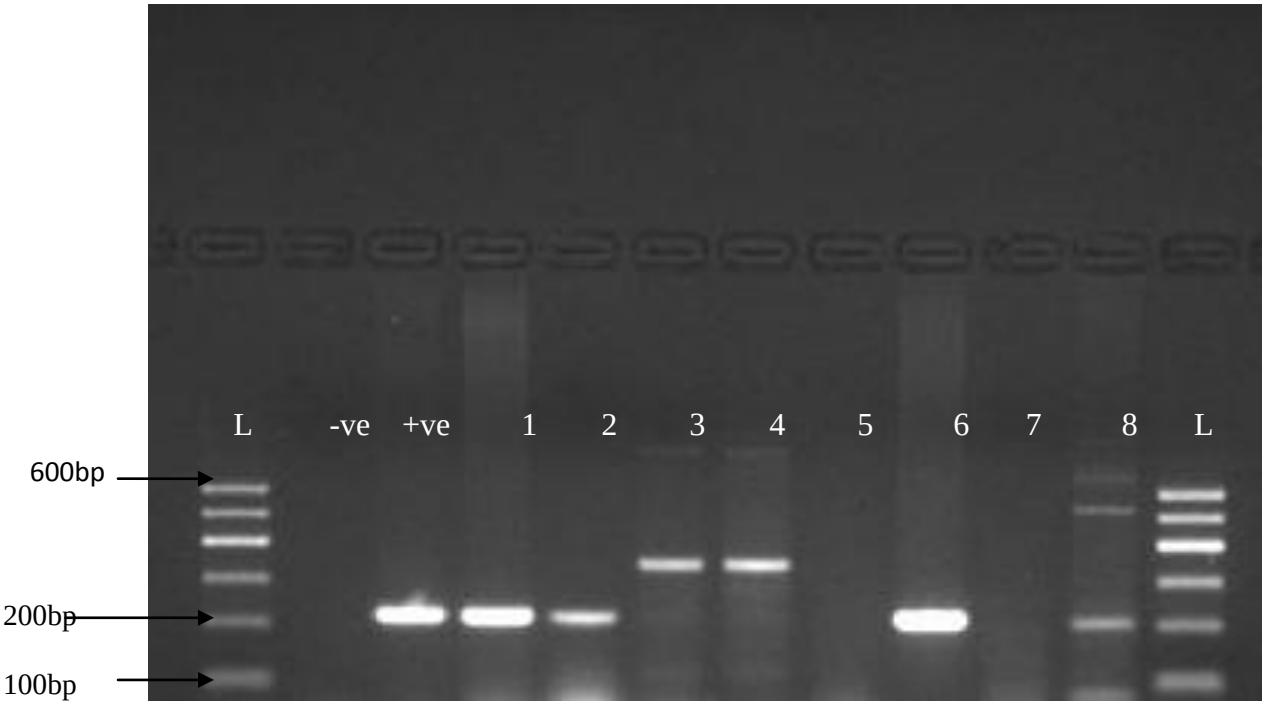


Plate V: Bands of 1% agarose gel of Polymerase chain reaction amplification product of Marek's disease virus Deoxyribonucleic acidfrom spleen samples using the Marek's disease virus serotpe 1 (RB-1B) specific primers, the positive band is at 200 bps -ve is the negative control, +ve is the positive control.

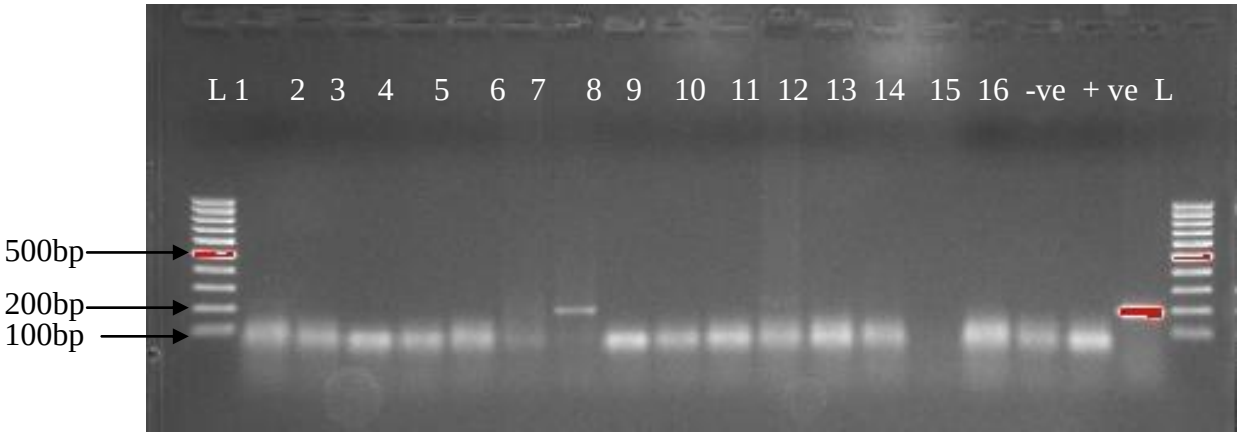


Plate VI:Bands of 1% agarose gel ofPolymerase chain reaction amplification product of spleen samples using the herpes virus of turkeys specific primers, the positive band is at 200bps -ve is the negative control, +ve is the positive control. Well 7 is positive , 1-6, 8-16 are negative.

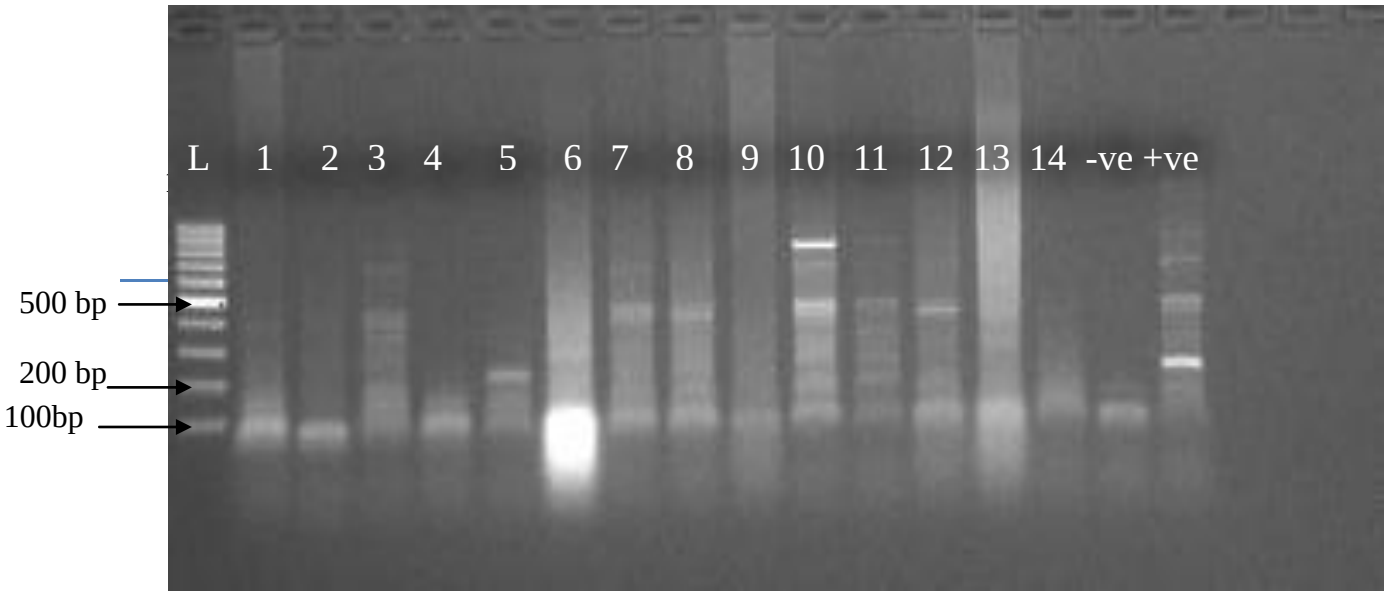


Plate VII: Bands of 1% agarose gel of Polymerase chain reaction amplification product of Marek's disease virus Deoxyribonucleic acid from spleen samples of layer using the Marek's disease virus serotype 1 pathogenic specific primers, the positive band is at 202 bps -ve is the negative control, +ve is the positive control. No 5 is positive, but 7,8,10 show multiple band.

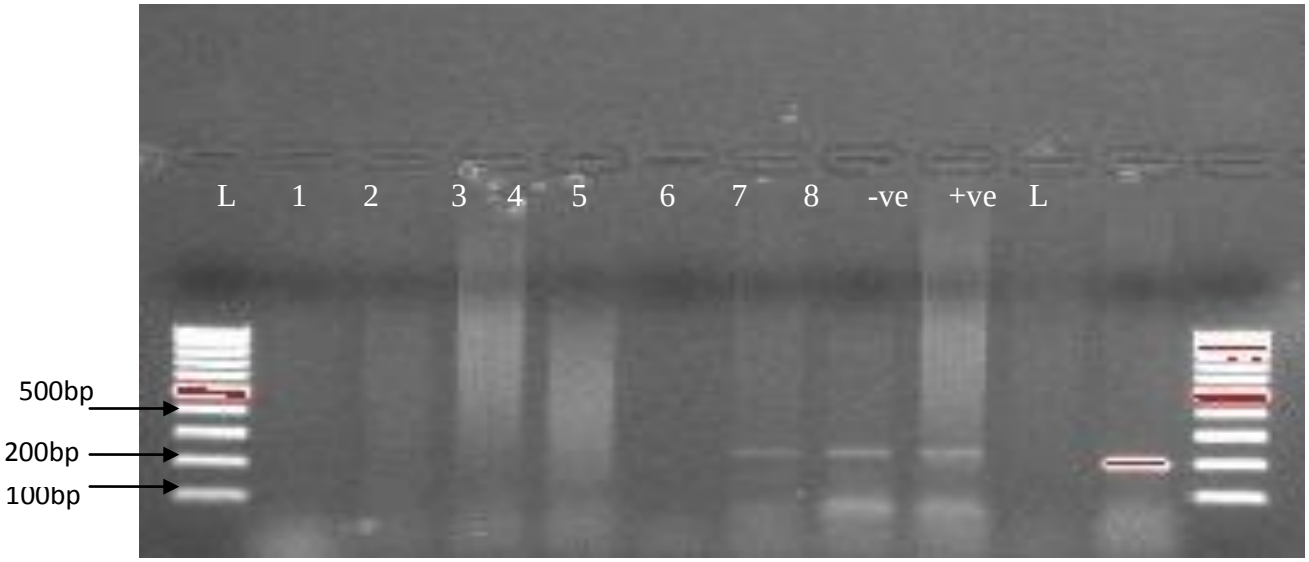


Plate VIII: Bands of 1% agarose gel of Polymerase chain reaction amplification product of Marek's disease virus Deoxyribonucleic acid of liver samples using the HVT FC 126 specific primers, the positive band is at 200 bps -ve is the negative control, +ve is the positive control. L is the ladder or marker, 1-5 are negative and 6-8 are positive.

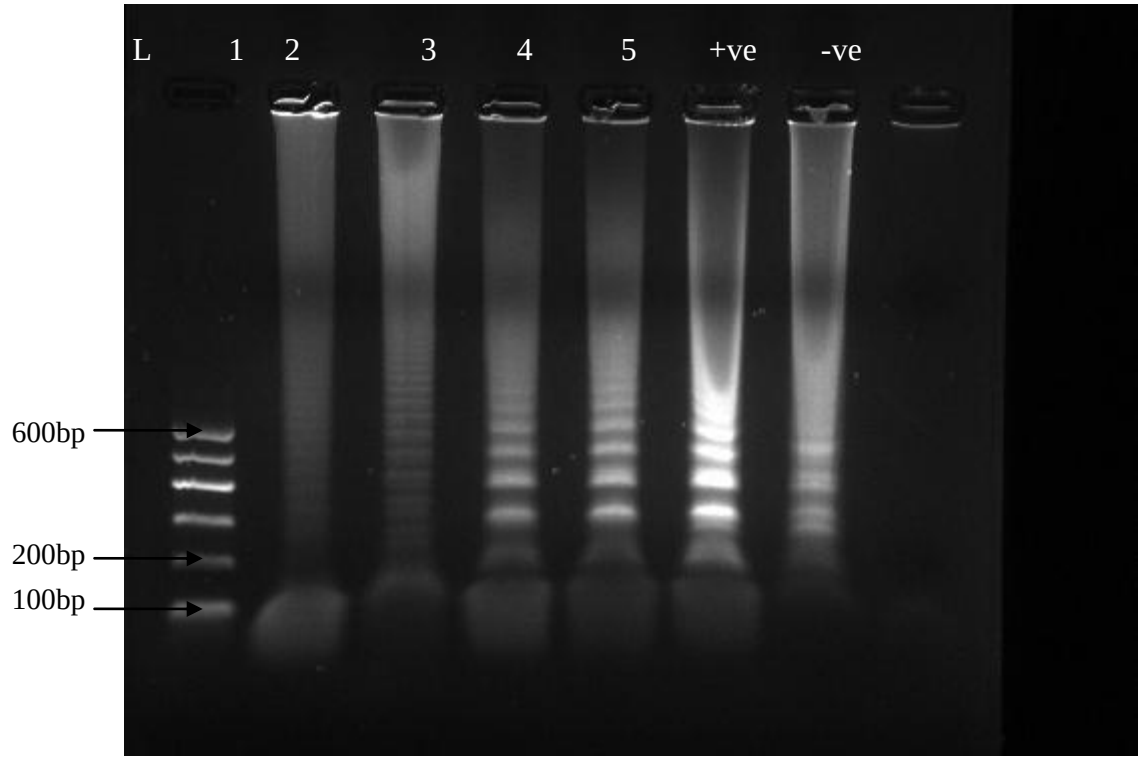


Plate IX**:**Bands of 1% agarose gel of loop mediated isothermal amplification procedure amplification product of Marek's disease virus Deoxyribonucleic acid using the herpes virus of turkeys specific primers, -ve is the negative control, +ve is the positive control 2-5 are positive. 1 is spleen of broiler, 2 is liver sample of broiler, 3&4 feather of layer 5 is skin of local chicken. L is the ladder.

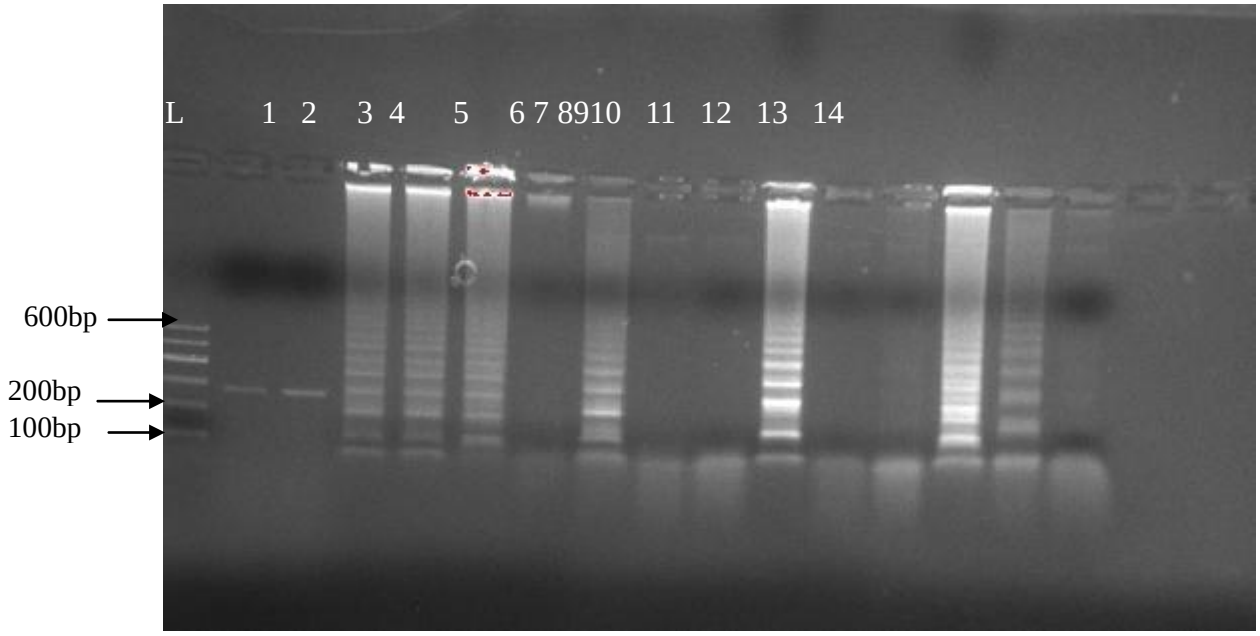


Plate X: Bands of 1% agarose gel of loop mediated isothermal amplification procedure amplification productof Marek's disease virus Deoxyribonucleic acid using the herpes virus of turkeys specific primers. 1-14 is different concentration of herpes virus of turkey FC 126 positive control.



Plate XI: Seventeen weeks old layer birds suffering from Marek's disease with uneven flock growth. Sample taken from the farm, Marek's disease virus serotype 1, RB-1B strain and Marek's disease virus serotype 1, Rispens strain were by polymerase chain reaction.

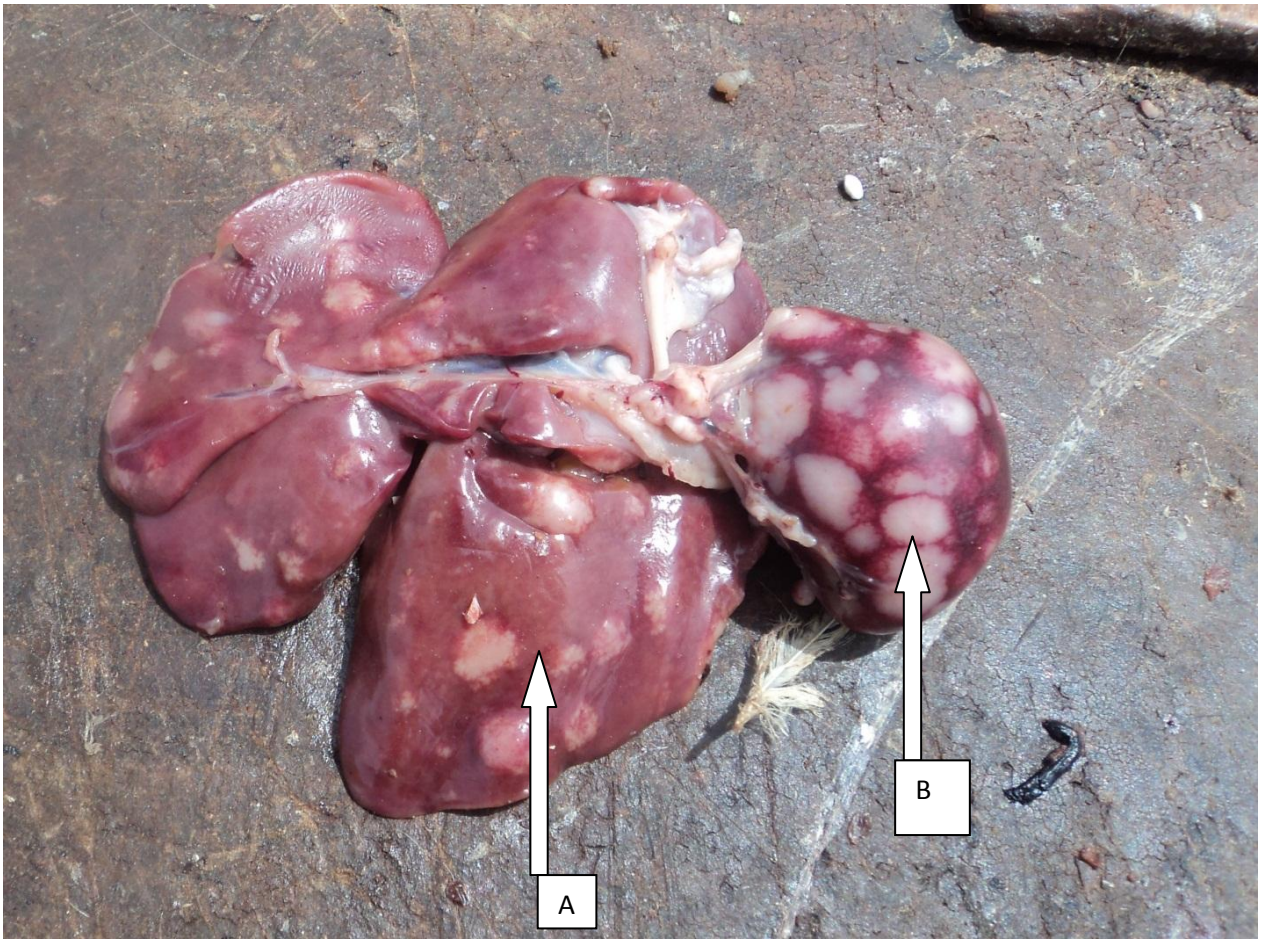


Plate XII: The liver (A) and spleen (B) of a 10 weeks old broiler showing gross lesions of multifocal nodular tumours. Marek's disease virus serotype 1 RB-1B strain was detected by polymerase chain reaction and confirmed by histopathology.

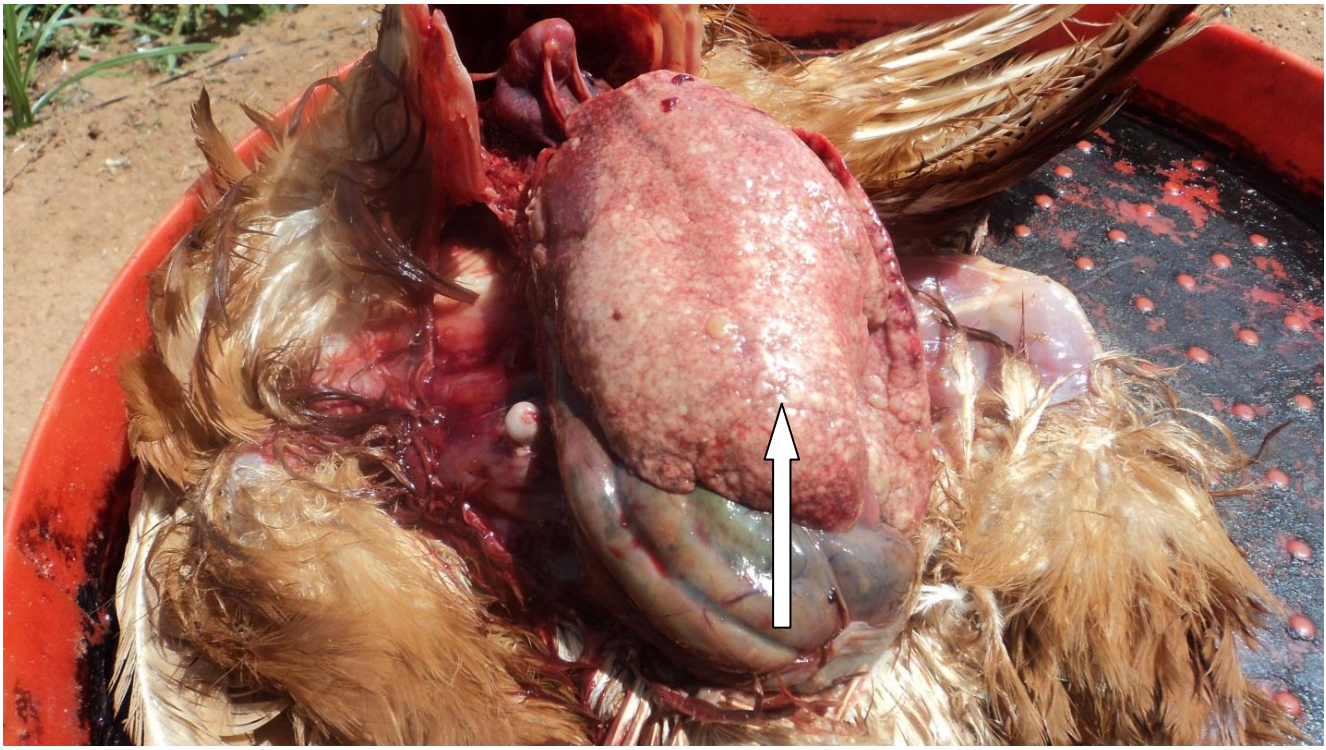


Plate XIII: Severely enlarged liver with numerous coalesced tumorous lesions in a layer bird. Marek's disease virus serotype 1 MB-1B strain and Marek's disease virus Rispens strain was detected by polymerase chain reaction in this sample.

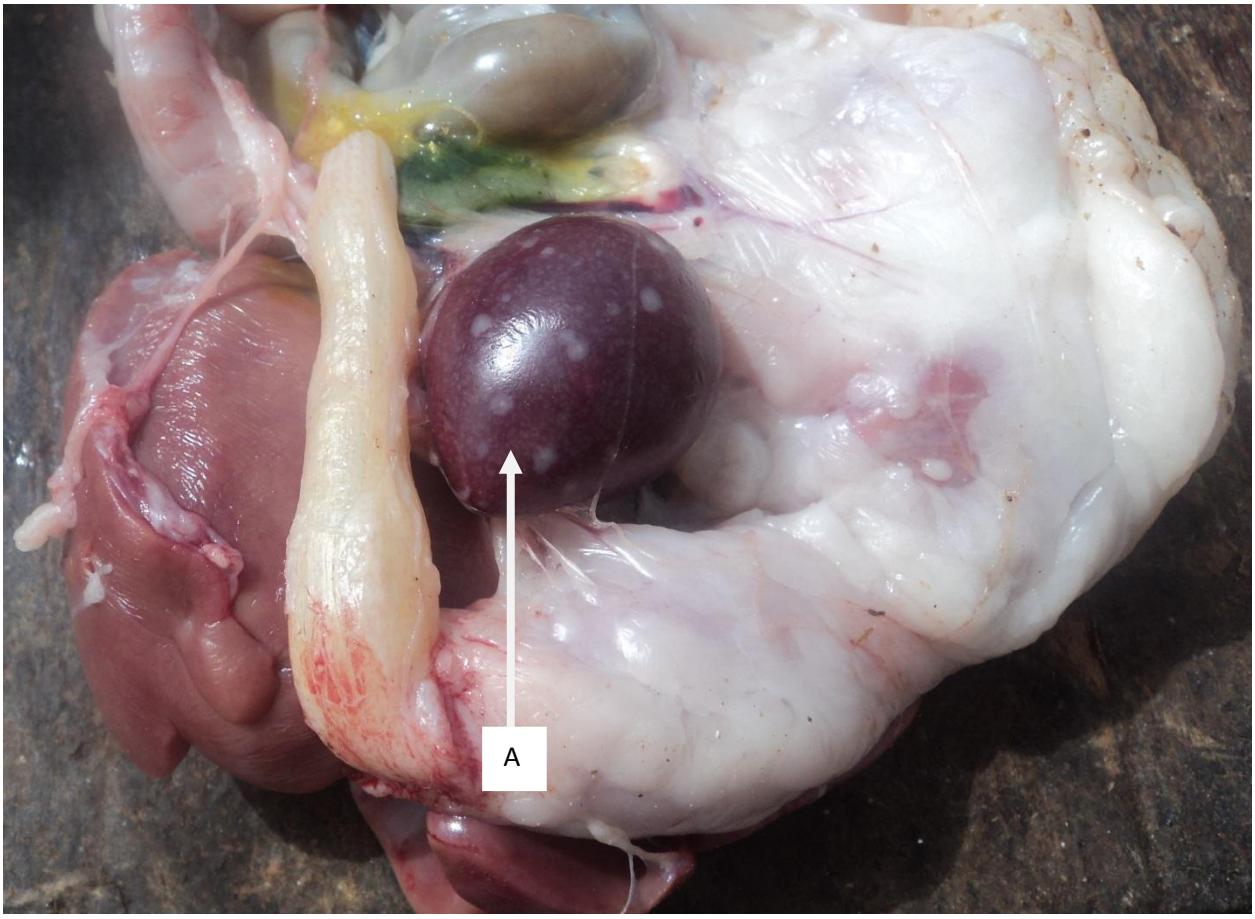


Plate XIV: Enlargement of the spleen with gray nodules(white arrow) in a nine weeks old broiler. Marek's disease virus serotype 1 RB-MB strain were detected by polymerase chain reaction.

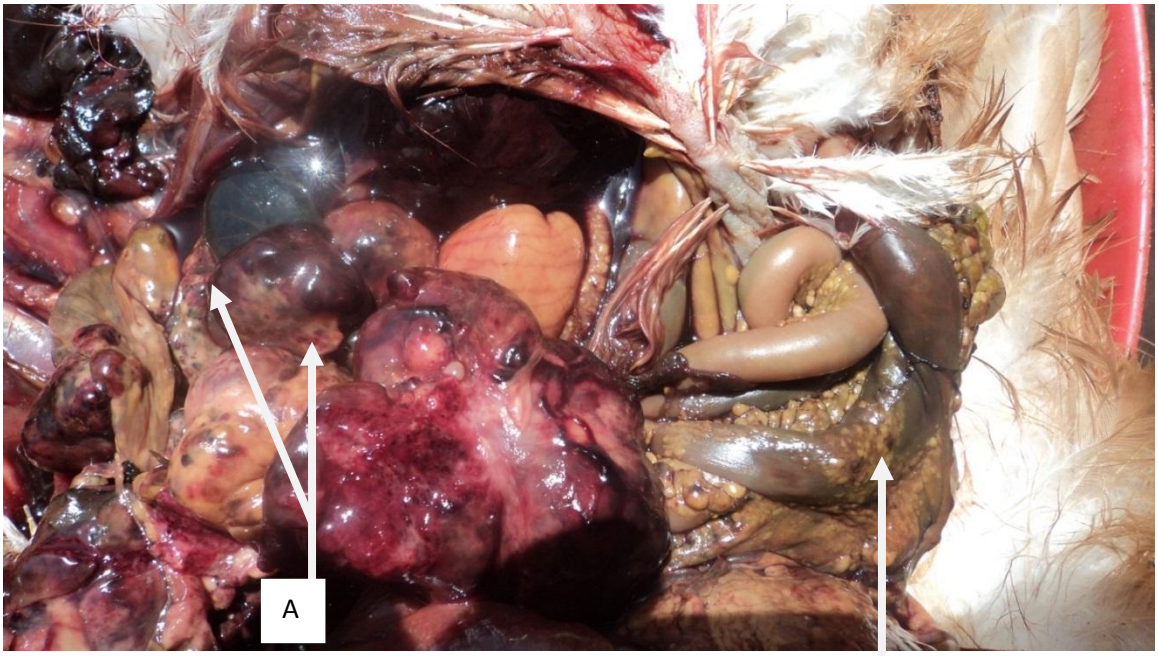


Plate XV: Viscera of a layer bird note tumorous lesions in the ov (A), intestine (B). Marek's disease virus serotype 1, MB-1B strain and Marek's disease virus Rispens strain was detected by polymerase chain reaction in this sample.

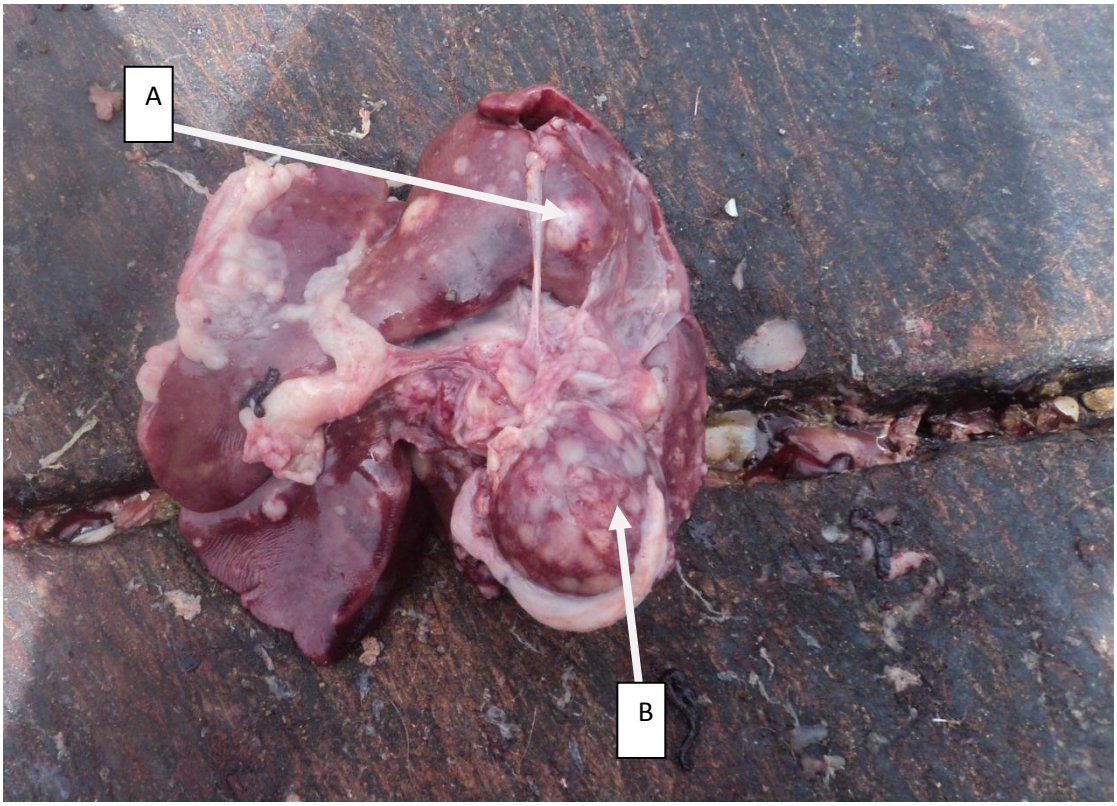


Plate XVI: The liver (A) and spleen (B) of a 10 weeks old broiler showing severe enlargement of the liver, spleen and with areas of gray nodules. Marek's disease virusMB-1B strain was detected by polymerase chain reaction in this sample. Marek's disease was also diagnosed by histopathology.

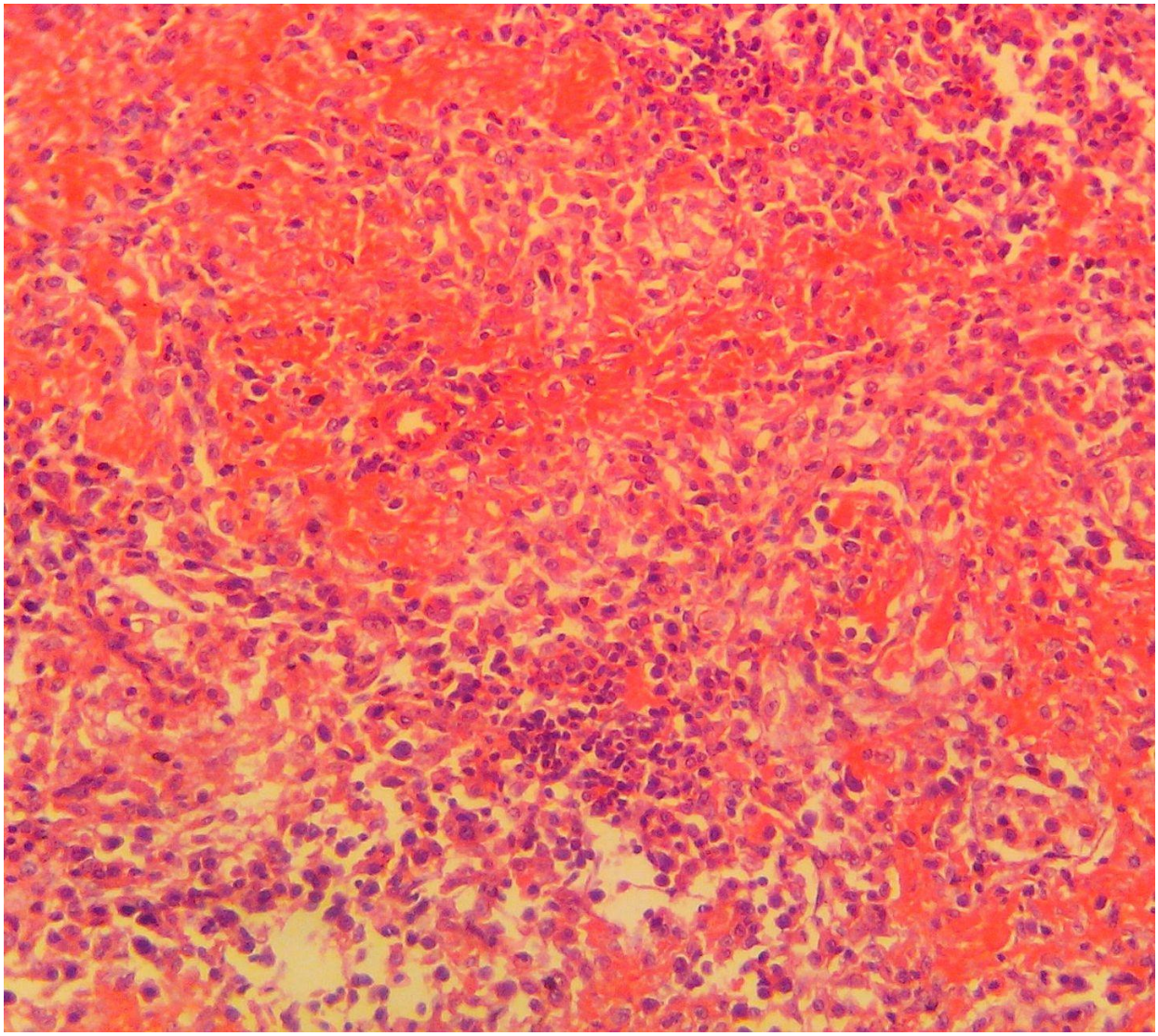


Plate XVII: Sample JNN201, spleen, diffuse infiltration of lymphohistioplasmacyticcellular infiltrates, with severe, diffuse haemorrhage occasionally forming nodules. (H&E X 400).

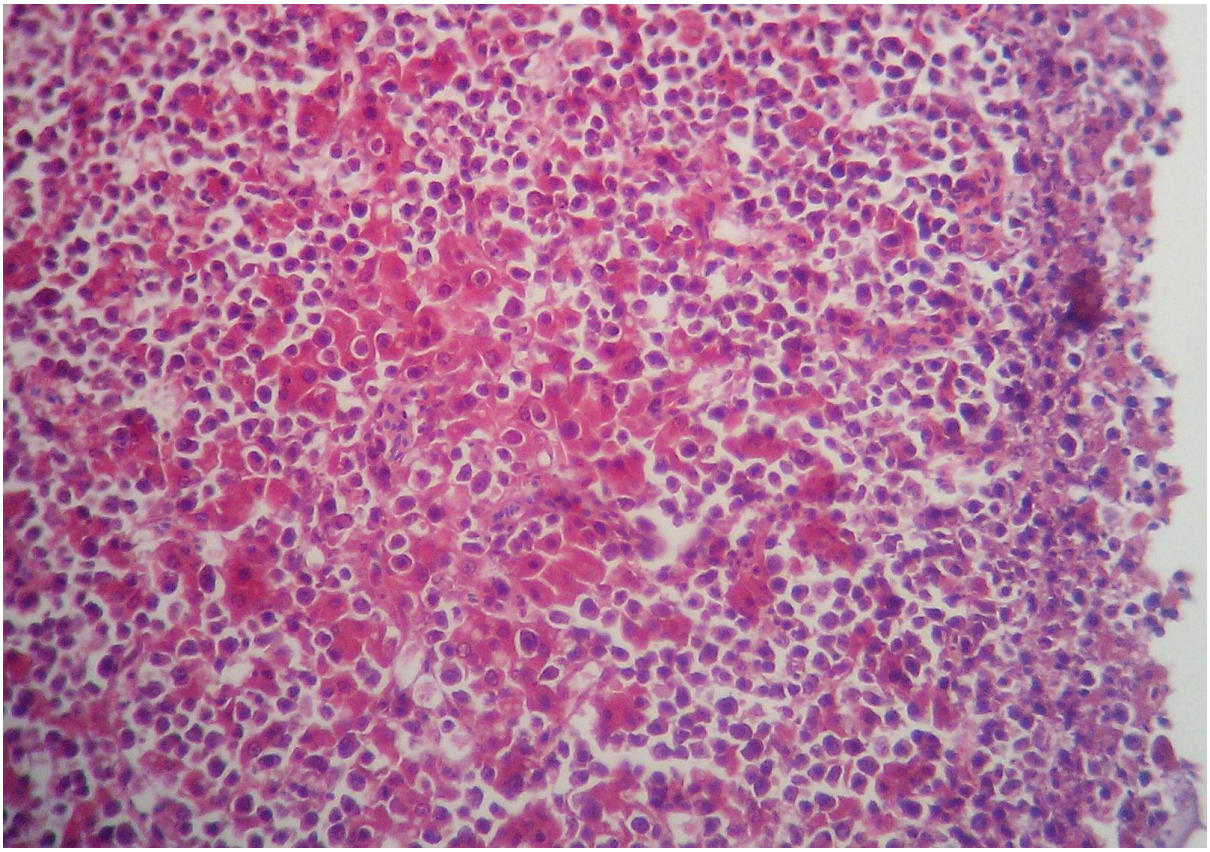


Plate XVIII:Liver (JNN 201)of 30 weeks layer, multifocal to diffuse hepatocellular necrosis and destruction of hepatic cords with lymphohistioplasmacytic cellular infiltration in inter-hepatic cord and sinusoidal spaces. H&E X 400.

CHAPTER FIVE

DISCUSSION

In this study, the retrospective prevalence of Marek's disease (MD) was 1.9% in Jos Plateau state, other findings in Nigeria reported higher prevalence rates of 60.6% for MD by Jwander *et al.*(2014), while Wakawa *et al.*, (2012) and Musa *et al.* (2013) reported prevalence of 8.3% and 4.9% respectively in Zaria.The reason for the low prevalence recorded in the study may be as result of lack accessibly to laboratory facility by some of the veterinary clinics in the study area, because diagnosis of MD was based on history, clinical signs and gross pathology. In addition results from the retrospective study shows that the prevalence of cases diagnosed as turmour was 10.9% compared to 1.99% for MD, some the cases may be MD, if further laboratory investigations were done or the veterinarians in the study area were better equipped to differentiate the various neoplastic diseases of poultry.

The retrospective study further reveals that March had the highest monthlyspecific rate of 2.96% of MD, although this finding differs from the reports of Wakawa *et al.*(2012) and Musa *et al.*(2013) in which the month of June had the highest monthly specific rate. The variance may be due to different location of the studies and the availability of the susceptible population of birds as result of different stocking time of chicks. Farmers in Jos, Plateau state stock chicks between December-January. While in Zaria common practice is to restock between March-May(Wakawa *et al.*,2012). Marek's disease was recorded in every month of the 7 years of retrospective study, suggesting that MD is endemic in Jos, Plateau State.

The yearly prevalence of the MD, showed that the highest prevalence recorded was in 2013, while other research findings byWakawa *et al.* (2012) and Musa *et al.* (2013) reported the highest prevalence in 2011 and 2009 respectively, furthermoreJwander *et al.*,(2014) reported the highest prevalence in 2008. All these points to the fact that MD is increasingly becoming a common disease of poultryin Nigeria,because cases of MDwere consistently reported between the year 2001 -2013,despite reports of routine vaccination of chicks.

This study also revealed that thedry season had the highest seasonal specific rate of MD and as earlier explained may be as result of large population susceptible poultry due to the common practice of restocking chicks between December and January.

In this study, thespecific rate of MD was highest in poultry above 18 weeks of agewhich agrees with the work done by Jwander *et al.* (2014). This finding is different from several reports that suggest MD mostly affects birds below 20 weeks of age. This can be attributed to the poor records kept at some of the veterinary clinics with not-specified age group (this group consist of birds in which the age and specie was not stated) having a high number of cases, some these cases may be MD of other age groups.

Marek's disease had the highest specific ratein layers compared to other poultry type which agrees with previous reports that female birds aremore predisposed to MD than male birds (Payne *et al.*,2000; Wakawa *et al,* 2012; Musa *et al.* 2013; Jwander *et al.*2014).In addition, MD was mostly diagnosed in exotic poultry as previously reported by Wakawa *et al.* (2012), Musa *et al.* (2013) and Jwander *et al.* (2014).

The prevalence of MD in broilers was 4.73% and cases diagnosed as turmour in broilers was 4.79% based on the retrospective study, although some of these cases diagnosed as tumour may be MD. Based on field observations in the study area, broilers are usually kept between 9-12weeksand alongside layers within the same premises. Marek's disease affect birds of all ages, particularly between 11-20weeks;this finding implies that MD may be an important disease of broilers, which is being enhanced by the practice of keeping broilers alongside with layers(Morrowand Fehler2004; Fadly and Nair 2008; Abdu and Usman, 2014). Broilers are not routinely vaccinated against MD in Nigeria, keeping them alongside with layers that are routinelyvaccinated exposes them to the field pathogenic and vaccine viruses which are horizontally transmitted(Fadly and Nair 2008). This further explains why different serotypes andstrains of MDV(MDV RB-1B 88%, MDV Rispens strain 86.6% and HVT FC126 Strain 16.67%) were detected in broilers by PCR during the study (Morrow *et al*., 2004; Wakenell *et al*., 2008)

Marek's disease is an immunosuppressive disease which destroys the immune system of poultry and hence increases thesusceptibility of the chickensto an array of infections with pathogens like *Escherichia coli, Emeria*and *Mycoplasma synoviae* (Morrow and Fehler, 2004;Schat, 2004; Gimeno, 2013). Findings from this study showed that MD was concurrently diagnosedwith colibacillosis, salmonellosis, Newcastle disease, coccidiosis, respiratory tract infection and fowl typhoid. Previous reports byIslam *et al.*,(2004) and Schat, (2004),observed that MD infection cause chickens to besusceptible to *E. coli* infection. Furthermore, from the findings of this study cases diagnosed to be tumour from the retrospective study were concurrently diagnosed with 14other different poultry diseases, which may indicate animmunosuppressive disease, MD inclusive. As a result of to the complexity of MD induce immunosuppression and its effects on

vaccination efficacy, it negative effects in the field is greatly underestimated and this effect can be exacerbated by the presence of other immunosuppressive factors like viruses and environmental factors (Gimeo, 2013).Marek's disease induced immunosuppression most likely leads to a lot of mis-diagnosis of MD and the poultry farmers' resource may be used for treatment of other infections without addressing the main underlining problem resulting in huge economic loses to the farmers.

One of theobjectives of this study was to enhance capacity for detectionof different serotypes and strains of MD viruses in Jos, Plateau State. Results of the analysisof the tumorous samples, showed thatthree strains MDV serotype 1 and one strain of MDV serotype 3 was detected by PCR in feathers, liver, spleen, heart, lungs, kidneys and skin samples as previously reportedPercentage detection rate was higher for liver than feathers and spleen which contradicts the findings of Davidson *et al.*(2002), the reason could because of the number of feathers and spleen samples analyzed were twice as much as the liver samples. It also confirms that feathers, liver, spleen, skin, heart, lungs and kidneys can be used for the detection of different serotypes and strains MDV in poultry(Davidson and Borenshtain, 2002;Owode and Oni, 2008; and Jwander, *et al.* 2012) Although feathers are still the preferred choice as sample for detection of MD virus as described in previous reports (Anagamuthu *et al.*, 2011; Wozniakowski *et al.*, 2011; Xiuying *et al.*, 2012). Because feathers samples recorded high detection rate compared to other samples apart from the liver and it is the site where the virus is shed (Davidson and Borenshtain, 2002). In addition non-invasive nature of sampling of feathers and they can easily be transported over a long distance with ease because of minimal cold chain requirement.

The PCR percentage detection rate of 87.90% forMDV RB-1B strain which is very virulent serotype 1 MDV indicate high number of cases of MDin Jos, Plateau State. The MDV RB-1B strain was detected in clinically diagnosed cases of MD with tumorous gross lesions of the visceral organs.Taking into consideration the case history, postmortem findings, histopathology and detection of very virulent MDV RB-1B strain, it confirms cases of MD in Jos, Plateau State (Burgess, 2003; Zelnik, 2004; Wozniakowski *et al.,* 2011; Singh *et al.,* 2012). The MDV RB-1B very virulent strain was detected by PCR in broilers, layers, quails and local chickens and it was also detected in birds of different ages which are new findings in Jos, Plateau State. All these results complemented with the retrospective study indicate that MD may be a common disease of poultry in Jos, Plateau State.

High detection rates were also recorded for vaccines strains of MDV particularly MDV Rispens strain in cases that the MDV RB-1B strains were detected. This suggests thatdespite routine vaccination at hatchery and revaccination twice at 3-21 days of age with MDV Rispens strain or HVT FC126 strain. The MDV RB-1B very virulent strain was detected and MD confirmed by histopathology in this study. The prevalence of MD all over the world had increased with the advent of vaccination against MD, which is similar to the finding in this study (Gimeno, 2008; Woziniakowski *et al.*, 2011). This is because, the MDV is becoming more virulence and the newer vaccines developed in the past forty years are not protective against very virulent strain of MDV. Another reason is that vaccination against MD does not confer sterilizing immunity, which implies that the field pathogenic MDV and vaccine MDV multiply simultaneously in the same bird and shed virus into the same environment with the possibility of exchange of genetic materials (Gimeno, 2008; Woziniakowski *et al.,* 2011).

The MDVRispens vaccine strain was detected in feathers, liver, spleen, and skin by PCR and this agrees with the work done by Islam *et al.*, (2007), Baigent *et al.*,(2005) and Gimeno *et al.,* (2012). These organs can be used for monitoring of MD vaccination as reported by Tan *et al.,* (2007), Cortes *et al.*,(2011) and Islam*et al.*,(2013). Monitoring MD vaccination is very important because of the myriad problems associated with vaccination against MD (Gimeno, 2008). This includes; poorly administered vaccines, the wrong type of vaccine, poor biosecurity practices and early chick exposure to pathogenic MDV (Gimeno, 2008).

This study is first to the best of my knowledge in Jos in which the MDV Rispens strain is detected by PCR, it is widely known that commercial poultry are routinely vaccinated in Nigeria at the hatchery as reported by Dunn and Gimeno,(2013) using the MDV Rispens strain which is the standard vaccine for the control of MD disease worldwide (Dunn and Gimeno, 2013). In Iraq, Waliq*et al.*(2013) reported prevalence of 46.7% and 48.1% in unvaccinated flocks of broilers andvillage chickens, respectively, while Baigent *et al.* (2013) in another study in Australia reported a prevalence 26% in unvaccinated flocks of broilers. The higher prevalence of MD in Iraq was as a result of poor biosecurity practices similar to the situation in Jos, Plateau State. In addition, detectionof the very virulent MDV RB-1B and the pathogenic Australian strains of MD in broilers, local chicken and quailssuggest that MD may be endemic in Jos, Plateau State. Furthermore, the practice of keeping other poultry like broilers, turkeys, quails and free range local chickens inclose proximity with commercial layers that are routinely vaccinatedexposes these birds to pathogenic MDV strains which are also shed into the environment.

According to the findings of Gimeno *et al.* (2012), for control of MD using vaccination it is better to use the combination of MDV Rispens + HVT strains as synergy for the best results, particularly when the birds are vaccinated at day 18 in-ovo and day 1 at the hatchery or within the first 21 days of age. Although MDV Rispens vaccine strain was detected by PCR, while HVT FC 126vaccine strain was detected by LAMP and PCR, the study was not able to confirm whether the MDV vaccine strain detected were as result of vaccination or the viruses were transmitted horizontally from contaminated environment.

Loop mediated isothermal procedure was difficult to optimize. The LAMP test was done over 13 times during the course of the study and acceptableresults were only obtainedtwice. However, LAMP detected MDV serotype 3 HVTFC 126 strain in liver, feathers and skin. The HVT FC 126 strain was also detected in broilers, layers and local chickens. The LAMP technique is simpler than PCR and reported to be more sensitive than PCR (Anagamuthu *et al.*, 2011; Wozniakowski *et al.*, 2011; Xiuying *et al.*, 2012). Unfortunately this study was not able to authenticate because of limitation of reagents. A lot of reagents were wasted in the process of optimization of the technique, but the major advantage of the technique is the use of simple equipment like the water bath for amplification of the DNA or RNA. A major challenge of field veterinarians observed during this study was lack of laboratory support in the diagnosis of MD and this can be overcome by further development and simplifying the LAMP technique to be used by these veterinarians.

Histopathology was used to further confirmed cases that the very virulent MDV RB-1B strain were detected by PCR and cases that PCR did not detect very virulent MDV RB-1B strain. The results revealed that 85.7% of positive samples were confirmed to be MD

by histopathology, while 24.3% of the negative samples were confirmed to be MD by histopathology. The reason for this variance may be due todifferent strains of circulating MDV in the poultry population in Jos, Plateau State from the primer sequences used to run the PCR. Histopathology was also used to further confirm MD in broilers, layers and local chickens in this study. This further burgesses the fact that MDis endemic in Jos, Plateau State, particularly diagnosis of MD in local chicken that are raised extensively will complicates the control of MD in Jos, Plateau State.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

- The overall prevalence of Marek's diseasein Jos, Plateau State was 1.99% between the years 2007-2013. Marek's disease was diagnosed in broilers (4.73%), layers (46.23%) and turkeys (0.72%).

- The study also showed that MD was concurrently diagnosed with coccidiosis (6.65%), colibacillosis (4.20%), salmonellosis (0.18%), Newcastle disease (8.41%), fatty liver syndrome (0.18%) and fowl typhoid (1.05%) in Jos, Plateau State.

- The detection rate of different serotypes and strains of MDV in Jos, Plateau State using PCR, was MDV Serotype 1(RB-1B)(87.90%), MDV Rispens strain (84.40%), MDV pathogenic strain (Australian strain) (7.94%) and HVT serotype 3 (FC 126) (9.09%). In addition MDV RB-1B strain was detected in feathers (90.38%), liver (100%), spleen (81.33%), heart (100 %), lungs (100%), kidneys (100%) and skin (100%). While MDV Rispens strain was detected in feathers (89.36%), liver (100%), spleen (78.95%) and skin (100%). The MDV pathogenic strain was detected in feathers (5.88%) andspleen (11.54%) while HVT serotype 3 (FC 126) was also detected in feathers (3.13%), liver (100%) and spleen (14.29%).

- The MDV RB-1B strain was detected in broilers (88.0%), layers (84.88%), local chickens (75%) and quails (100%), while MDV Rispens strain was detected in broilers (86.67%), layers (83.52%), local chickens (50%) and quails (100%). Furthermore, MDV pathogenic strain was detected only in layers (12.5%) and HVT serotype 3 was detected in broilers (16.67%), layers (12.50%) and local chickens (33.33%).

- Samples positive by PCR (85.7%) for MDV RB-1B were also positive by histopathology and gross examination. Some samples had gross lesions suggestive of MD and confirmed to be MD by histopathology were negative by PCR (24.3%) for MDV RB-1B strain.

- Loop mediated isothermal procedure was used to detect HVT FC 126 in liver (100% 1/1), feathers (16.6%, 1/6) and skin (100% 1/1). In addition, HVT FC 126 was detected in broilers, layers and local chickens.

6.2 Recommendations

- More work should be done by Research Institutes and Veterinary Schools to fully understand the epidemiology and diagnosisof the MD in Jos and other parts of Nigeria.

- Field veterinarians should improve record keeping of clinical cases and criteria of differentiating MD from other neoplastic diseases of poultry so that the actual prevalence of MD in Jos, Plateau State will be known.

- Researchers in Research Institutes and Universities need to understand and optimize the use of LAMP which is cheaper, faster and more sensitive than PCR so that it can be used by small laboratories for confirmatory diagnosis of MD (particularly feathers that can be collected using non-invasive methods and transported without cold chain) in Nigeria

- Since the MD virus was detected in the present study, it is recommended that researchers in Research Institutes and Universities should characterize the circulating serotypes and strains of MD virus in Jos.

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Appendix I: Post mortem examination form

POSTMORTEM EXAMINATION FORM

History

Date		Case No.	
Name of Owner/ Farm			
Address			
Source of Birds	Breed		Sex
Age of Birds		Population Of Birds	
Source of Feed			
Date of Disease Onset			
Egg Production History			
No Presented For Examination		No. dead	
Vaccination History			
Clinical Sign			

Post Mortem Findings

Air Sac	Liver
G/Bladder	Heart
Lungs	Spleen
Ovarian Follicles	Kidneys
Oviduct	Testes
Brain	Nerves
Trachea	Esophagus
Crop	Proventriculus
	Pancreas
Ventriculus	
	Jejunum
Duodenum	
Ileum	Ceca
Ceca tonsils	Rectum
Others	

Type of specimen collected

Appendix II: Yearly distribution of tumour cases diagnosed in poultry from Veterinary clinics and diagnostic centre in Jos, Plateau State (2007-2013).

Year	Tumour (%)	NTM	Total cases	Odds ratio	CI	p-value
2007	123 (6.38)	1,805	1,928	1		
2008	426 (10.27)	3,724	4,150	1.67	1.36-2.07	0.0001
2009	988 (13.34)	6,420	7,408	2.26	1.85-2.74	0.0001
2010	597 (12.02)	4,369	4,966	2.01	1.64-2.45	0.0001
2011	763 (12.24)	5,470	6,233	2.05	1.68-2.45	0.0001
2012	252 (8.05)	2,878	3,130	1.29	1.028-1.61	0.026
2013	2 (0.22)	906	908	0.03	0.008-0.13	0.000
Total	**3,151 (10.99)**	**25,572**	**28,723**			

Appendix III: Monthly distribution of tumour cases diagnosed in poultry from veterinary clinics and a diagnostic centre in Jos , Plateau State (2007-2013)

Month	Tumour (%)	Non tumour cases	Total cases	Odds ratio	Confidence interval	p-value
Jan	174 (10.79)	1,438	1,612			
Feb	238 (11.82)	1,776	2,014	0.90	10.37-12.44	0.335
March	381 (13.82)	2,352	2,733	0.75	11.81-13.8	0.002
April	358 (12.12)	2,597	2,955	0.88	10.75-12.61	0.183
May	361 (10.72)	2,990	3,351	1.00	9.94- 11.67	0.978
June	410 (12.57)	2,851	3,261	0.84	11.10-12.93	0.070
July	339 (12.88)	2,293	2,632	0.82	11.14-13.10	0.042
August	262 (9.78)	2,417	2,679	1.12	9.29-11.10	0.287
Sept	187 (9.12)	1,863	2,050	1.21	8.93- 10.87	0.093
Oct	173 (8.24)	1,926	2,099	1.35	8.45-10.33	0.008
Nov	140 (7.08)	1,838	1,978	1.59	7.86- 9.716	0.000
Dec	128 (9.42)	1,231	1,359	1.16	9.12-11.30	0.217
Total	**3,151(10.99)**	**25,572**	**28,723**			

Appendix IV: Seasonal distribution of tumour cases diagnosed in poultry from veterinary clinics and diagnostic centre in Jos, Plateau State (2007-2013).

Season	Tumour (%)	Non tumour cases	Total cases	Odds ratio	Confidence interval	p-value
Dry (Jan-March)	793 (14.2)	5,566	6,359			
Pre rainy (Apr-June)	1129 (13.37)	8,438	9,567	1.06	11.57-12.58	0.204
Rainy (July-Sept)	788 (11.98)	6,573	7,361	1.18	11.00- 12.07	0.001
Pre dry (Oct-Dec)	441 (8.8)	4,995	5,436	1.61	9.92- 11.03	0.000
Total	**3,151(10.99)**	**25,572**	**28,723**			

Appendix V: Age distribution of tumour casesdiagnosed in poultry from veterinary clinics and a diagnostic centre in Jos, (2007-2013)

Age group in weeks	Broiler(%)	Layer(%)	Turkey(%)	Geese(%)	Not specified (%)	Total (%)
0-6	25(0.79)	47(1.49)	0	0	0	72(2.28)
>6-18	45(1.43)	342(10.85)	0	0	0	387(12.28)
18>	0	602(19.11)	0	0	0	602(19.11)
Not-specified	81(2.57)	733(23.26)	2(0.06)	1(0.03)	1273(40.40)	2090(66.33)
Total	**151(4.79)**	**1724(54.71)**	**2(0.06)**	**1(0.03)**	**1273(40.4)**	**3151(100)**

Appendix VI: Distribution of concurrent infection of tumour cases and other diseases diagnosed in poultry from veterinary clinics and a diagnostic centre in Jos, Plateau State (2007-2013).

Disease condition	Layer(%)	Broiler(%)	Turkey(%)	Geese (%)	Not Specified (%)	Total
Tumour	1260(73.09)	95(62.91)	2(100)	1(100)	1113(87.43)	2471(78.4)
Tumour + Salmonellosis	10(0.58)	1(0.66)	0	0	6(0.47)	17(0.54)
Tumour + Colibacillosis	100(5.80)	8(5.30)	0	0	50(3.93)	158(5.01)
Tumour + ND	79(4.58)	18(11.92)	0	0	0	111(3.52)
Tumour + Malnutrition	6(0.35)	1(0.66)	0	0	0	7(0.22)
Tumour + Coccidiosis	78(4.52)	10(6.62)	0	0	0	116(3.68)
Tumour + ND+ Coccidiosis	13(0.75)	3(1.99)	0	0	1(0.08)	17(0.54)
Tumour + Salmonellosis+ Colibacillosis	3(0.17)	0	0	0	0	3(0.10)
Tumour + FLS+ FT	2(0.12)	0	0	0	0	2(0.06)
Tumour + Bacterial Infection	90(5.22)	14(9.27)	0	0	5(0.39)	109(3.46)
Tumour +FLS	19(1.10)	0	0	0	0	19(0.60)
Tumour + Colibacillosis + ND	4(0.23)	0	0	0	6(0.47)	10(0.32)
Tumour +FT	26(1.51)	0	0	0	2(0.16)	46(1.46)
Tumour + Helminthosis	19(1.10)	0	0	0	7(0.55)	26(0.83)
Tumour + Coccidiosis+ Colibacillosis	5(0.29)	0	0	0	2(0.16)	8(0.25)
Tumour + CRD	9(0.520	0	0	0	10	19(0.60)
Tumour + Aspegillosis	1(0.05)	0	0	0	0	1(0.03)
Tumour + ND + CRD	0	0	0	0	6	6(0.19)
Tumour + Pullorum	0	0	0	0	1(0.08)	1(0.03)
Tumour + Fowl Cholera	0	0	0	0	2(0.16)	2(0.06)
Tumour + Fowl Pox	0	0	0	0	2(0.16)	2(0.06)
Overall total	**1724(54.71)**	**151(4.79)**	**2(0.06)**	**1(0.03)**	**1273(40.40)**	**3151(100%)**

Appendix VII: **Distribution of unspecified tumour cases by specific age of poultry in Jos, Plateau State (2007-2013).**

Age in weeks	Broilers	Layers
1	0	1
2	3	3
3	1	0
4	1	6
5	8	2
6	12	1
7	7	6
8	17	28
9	7	12
10	5	17
11	4	27
12	3	55
13	1	32
14	0	39
15	0	40
16	1	64
17	0	56
18	0	40
19	0	38
20	0	67
21	0	28
22	0	39
23	0	24
24	0	46
25	0	21
26	0	23
27	0	11
28	0	58
29	0	9
30	0	11
31	0	7
32	0	43
33	0	3
34	0	1
35	0	4
36	0	17
37	0	1
38	0	3
40	0	25
44	0	15
45	0	3
51	0	1
52	0	42
53	0	3
56	0	3
58	0	1
60	0	5
68	0	1
72	0	5
78	0	1
80	0	3
Total	70	991